HUMAN EXPOSURE TO FOODBORNE TOXINS IN GHANA: INTERVENTION STRATEGY FOR REDUCTION OF AFLATOXIN AND FUMONISIN BIOAVAILABILITY

A Dissertation

by

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ABSTRACT

International health has typically focused on remediation of infectious diseases in developing countries. However, recent reports from the International Agency for Research on Cancer (IARC) have highlighted the importance of cancer incidence/ mortality in the developing world. Foodborne mycotoxins produced by fungi, called aflatoxin (AF) and fumonisin (FB), have been associated with hepatocellular and esophageal carcinomas among other deleterious effects, such as growth faltering and immune dysfunction. Exposure to these toxins in Ghana is particularly high due to food insecurity, climate, and lack of regulatory infrastructures. Work to alleviate AF and FB contamination in Africa has focused on instituting good agricultural and storage practices however, exposures remain inextricable in many communities. Utilization of a calcium montmorillonite clay, UPSN, shows promise of tightly binding both AF and FB in the gastrointestinal tract, thereby reducing their bioavailability. The objectives of this research were to determine exposure susceptibility in Ghana and to assess efficacy and safety of UPSN treatment within vulnerable populations.

Cross-sectional data from six different regions of Ghana indicated that AF exposure is associated with maize consumption and region of residence. However, food preparation practices were not correlated to AF levels in the present study. Therefore, future intervention strategies were focused on the end point of the food consumption chain by reducing AF exposure from maize immediately prior to ingestion (i.e. UPSN treatment). In a three-month trial an encapsulated montmorillonite clay was efficacious in reducing AF exposure. However, concern for sustainability and its applicability for

children led to an effort to alter the dose dissemination form. Inclusion of UPSN in common Ghanaian foods retained the efficacy of the clay, reducing a short-term biomarker (AFM_1) by 55%, and was determined to be safe in children (ages 3-9). Importantly, daily assessment of AFM_1 levels was successful in providing statistical significance of intervention effects within only five days of treatment. Initial results indicate that UPSN could efficiently to bind both AF and FB in the gastrointestinal tract, reducing biomarkers for both toxins in animal models. Thus, UPSN could positively impact health in developing communities at risk for AF and FB exposure.

DEDICATION

To my family and friends who always lent an ear when I was frustrated, had a joke when I was overwhelmed, put things in perspective when I was feeling lost, and believed in me every step of the way.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

1. INTRODUCTION

Worldwide cancer patterns have been changing drastically over the past decade. Cancer was previously considered to be a more prevalent disease in the developed world; however, the cancer incidence and mortality in developing countries has been rising. The *World Cancer Report* (2002), produced by the International Agency for Research on Cancer (IARC), clearly delineates the rising concern for worldwide public health care expenses, mortality, and morbidity resulting from cancer incidence (Parkin et al. 2005). In 2002, global mortality from cancer was higher than that from HIV/AIDS, tuberculosis, and malaria combined; accounting for 7.6 million lives lost. Following current trends, it is estimated that cancer burden will increase from 10 million new cases per year in 2000 to 16 million in 2020; with the largest burden (70%) occurring in the developing world (Lingwood et al. 2008). Annual new cases of cancers in developing countries are highest among lung and bronchus, stomach, liver, colon, and esophagus for men (Ferlay et al. 2010). Breast, cervical, lung and bronchus, stomach, colon, and liver make up the primary cancer types in women from the developing world (Ferlay et al. 2010). In particular, more than 1 million of these cases are suspected to occur in Sub-Saharan Africa (Parkin et al. 2003). The risk of dying from cancer in Africa is almost double that of developed countries due to late-onset diagnosis, inadequate treatment facilities, and few effective cancer medicines that are affordable and do not require hospitalization (Lingwood et al. 2008; Parkin et al. 2008). Adoption of a western

lifestyle, longer life, and improved diagnosis has been implicated in the rise of cancer incidence observed in Sub-Saharan Africa (Jemal et al. 2011).

Primary liver cancer is of particular interest in these populations as it typically carries a very poor prognosis and remains the second and third highest in incidence and mortality in men and women in Sub-Saharan Africa, respectively (Ferlay et al. 2010; Parkin et al. 2005). The median survival rate of primary liver cancer patients is less than one year (Nguyen et al. 2009). More than 80% of the liver cancer cases occur in the developing world. The highest risk is in populations of Africa and Asia due to various factors; including a high prevalence of Hepatitis B and C (HBV and HCV) infection, alcohol consumption, dietary aflatoxin (AF) exposure, tobacco smoking, obesity, fatty liver, and iron load (Parkin et al. 2005). Of these risk factors HBV infection and exposure to a carcinogenic mycotoxin, named AF, are the two variables primarily implicated in the development of hepatocellular carcinoma (HCC) in Africa and Asia (Parkin et al. 2005). HCC accounts for 80% of all primary liver cancer cases in the world and is attributed to the unregulated growth and cellular replication of hepatocytes. HBV transmission in Africa occurs typically through an unexplained route of horizontal transmission between toddlers, with exposure and subsequent infection arising before the age of 5 (Burnett et al. 2012). Association of HCC incidence with HBV occurs in individuals who are chronically infected, thus consistently testing positive for HBV surface antigen (HBsAg). Although, introduction of HBV vaccination programs are currently underway in Africa and Southeast Asia it will take many generations to positively affect change in HCC incidence. Moreover, it is estimated that 40% of the

HCC cases attributable to AF exposure alone, occur in Africa (Liu Y and Wu 2010) and thus would not be affected by HBV vaccination. Other factors contributing to HCC rates in Western Africa include similar environmental carcinogens, such as fumonisin (FB) mycotoxins. Therefore, the development of interventions that are economically feasible, culturally acceptable, and would be sustainable in rural African communities are important to help reduce the burden of mycotoxin-induced health effects.

1.1 Aflatoxin

1.1.1 Aflatoxin chemistry

Since the discovery of AFs in the early 1960s there has been a constant interest in the toxic effects and prevention of AF exposure in animals and humans. The AF congeners were isolated, and characterized chemically in the lab of Dr. Gerald Wogan in 1963 following reports of acute hepatotoxicity in turkey poults in England (Asao et al. 1965; Blount 1961; Lancaster et al. 1961). Exposed animals all had similar physiological endpoints of toxicity including acute hepatic necrosis, bile duct hyperplasia, loss of appetite, wing weakness, and lethargy (Blount 1961). Reports of turkey "X" disease causing deaths of thousands of chickens, turkeys, and ducks were later linked to consumption of contaminated feeds containing high levels of peanut meal as a protein source (Cullen and Newberne 1994). The discovery of toxicity resulting from the feed led to the hypothesis that AFs were an environmental contaminant. Further research indicated that AFs were indeed products of certain fungal strains that commonly grow on grains and groundnuts, such as peanuts. Subsequently, AFs were then assessed for toxicological significance in multiple laboratory animal species. Although species

differences in susceptibility were apparent, the target of AF toxicity has been proven to be the liver in most animals (Cullen and Newberne 1994).

AFs comprise one group of over 300 known mycotoxins (Shephard 2006) and exposure predominantly occurs through ingestion of foods or feeds contaminated with *Aspergillus flavus* and/or *A. parasiticus* fungi. These fungi can produce four structurally different congeners of AF: B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2). Structurally, AFs are highly substituted coumarins containing a fused dihydrofuran moiety. AFB_1 and AFB_2 are so named due to an emission of blue fluorescence following ultraviolet light stimulation and are characterized by the fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety. AFG_1 and AFG_2 emit a greenish yellow fluorescence under ultraviolet light and contain a fused lactone ring in the coumarin moiety (Figure 1). AFB_1 and AFG_1 are the most toxic due to an unsaturated double bond at the 8,9 position on the terminal furan ring which can be metabolized in animals and humans to a toxic and mutagenic epoxide.

1.1.2 Source of aflatoxin contamination

Aspergillus flavus and *A. parasiticus* can inoculate and colonize different crops by three primary routes: 1) insect transfer, 2) airborne spores, and 3) soil contact. Maize crops are one of the major hosts for *A. flavus* and *A. parasiticus* colonization. It was first determined by Anderson et al. (1975) that these pathogenic fungi could colonize maize not only during storage, but also in the field. The main route of inoculation of the fungi occurs through the silk by insects acting as vectors between contaminated ears. It has been suggested that insects facilitate infection in pre-harvest maize by transporting

Aspergillus spores from silk to silk, disseminating inoculum within the ears, and creating a favorable environment for *Aspergillus* growth and colonization through injury of the protective pericarp of maize kernels (Marsh and Payne 1984; Payne 1992; Wilson and Payne 1994). Peanut crops, like maize, are a favorable host for *Aspergillus*, however the route of contamination most likely originates from the soil, as the fruit of this plant develops underground (Cole et al. 1989). Contamination of both crops may also occur through airborne spores inoculating the silk of the maize and the above ground flower of peanut plants as well (Griffin and Garren 1976; Wilson and Payne 1994). It is important to note that crops infected with *Aspergillus* fungi do not always contain AFs. Production of mycotoxins by different fungal species depends largely on the environment in which the fungi are growing. In particular, the temperature and moisture content during *Aspergillus* growth have an effect on production of AFs during both pre-harvest growth and post-harvest storage. Climates where temperatures are predominantly below 20°C tend to have a low likelihood of *Aspergillus* contamination, while tropical climates (i.e. temperature minimum is >25°C) have common contamination in the soils, air, and crop surfaces (Cotty and Jaime-Garcia 2007a; Shearer et al. 1992). Rises in temperature during drought, in tropical countries with semi-arid and arid climates, has been associated with increases in levels of AF contamination of developing crops (Sanders et al. 1984; Schmitt and Harburgh Jr. 1989). Following contamination of developing crops with *Aspergillus* species; warm, moist conditions (i.e. high humidity) can increase production of AFs in mature crops (Cotty 1991; Russell et al. 1976). High humidity results in a high water content of previously dry seeds, which is conducive to

contamination and fungal growth. This effect has been best portrayed by Jaime-Garcia and Cotty (2003), where AF contamination was highest when mature crops were rained on just prior to or during harvesting. The influence that climate has on AF production results in continued exposure in human populations inhabiting tropical and sub-tropical areas.

1.1.3 Aflatoxin toxicity

The toxicity of AFs has been exhaustively studied in multiple animal species. While AFB_1, AFB_2, AFG_1, and AFG_2 are considered the "major" congeners from A. flavus and A. parasciticus, AFB_1 is the most toxic and prevalent in food-stuffs (CAST 2003). Acute toxicity from AFB_1 exposure, termed aflatoxicosis, typically involves symptoms of anorexia, depression, ataxia, dyspnea, anemia, and hemorrhaging from body orifices; these conditions are often followed by death. Sensitivity to AFB_1 has been established in vertebrates, invertebrates, plants, and bacteria; however the range of toxin dosimetry is wide. Differences in species susceptibility could be attributed to three important metabolic factors: 1) activation of AFB_1 to the toxic 8,9 epoxide relative to other less toxic metabolites, 2) conjugation pathways resulting in relatively nontoxic metabolites that are eventually excreted, and/or 3) differences in absorption from the gut. As previously stated, the metabolic activation of AFB_1 across the 8,9 double bond forms a short-lived but highly reactive epoxide that is the principle mediator of cellular injury.

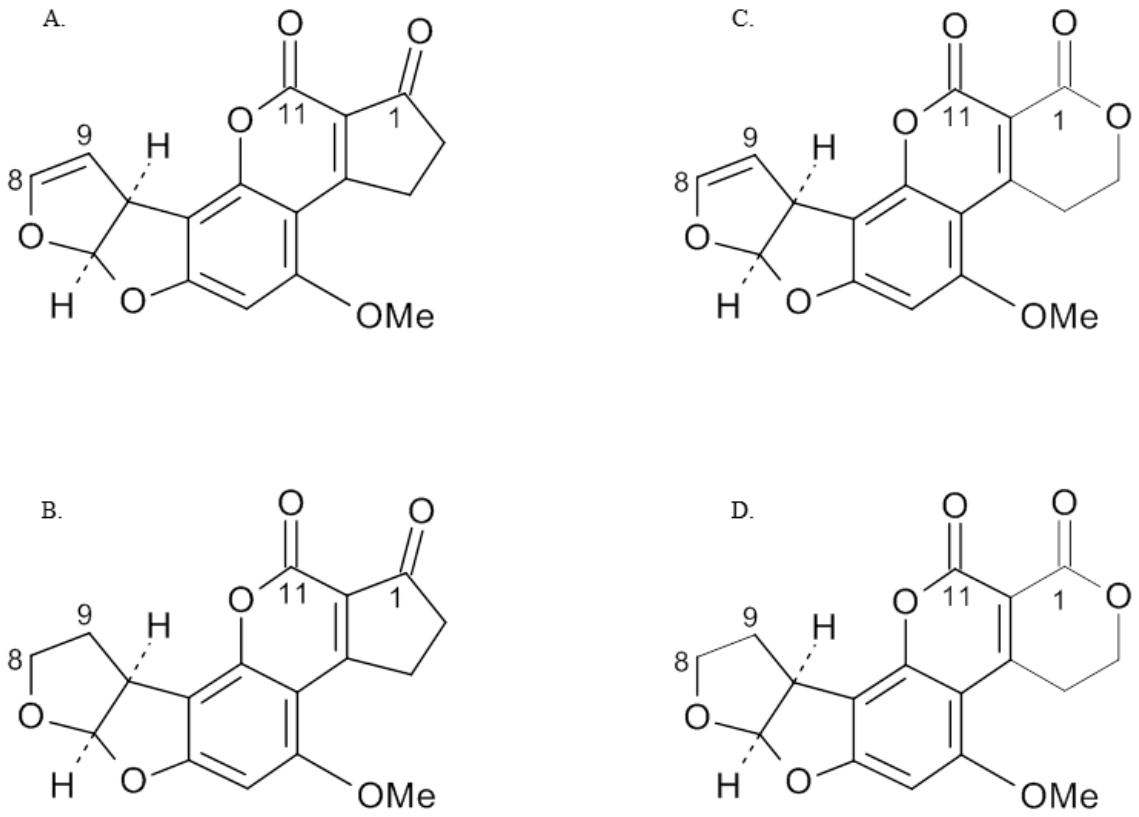


Figure 1. Chemical structures of the four congeners of naturally occurring aflatoxins. A) Aflatoxin B_1 (AFB_1). B) Aflatoxin B_2 (AFB_2). C) Aflatoxin G_1 (AFG_1). D) Aflatoxin G_2 (AFG_2). The B series are so named for emitting blue fluorescence and the G series emit green fluorescence under ultraviolet light.

The LD_{50} value for acute toxicity among non-human species ranges from 0.3-18.0 mg/kg, depending on species, sex, route of administration, and age difference (Cullen and Newberne 1994).

1.1.4 Carcinogenicity

Following discovery of acute toxicity mechanisms and doses in animals it became evident that AFB_1 was hepatocarcinogenic. Variability in the LD_{50} values among species is important not only in determining acute susceptibility, but also in indicating species susceptibility for cancer development. For instance, some of the most sensitive species to AFB_1 are ducks and rabbits, while chickens are highly resistant. Chickens appear to be resistant to AF-induced carcinoma (Roebuck and Maxuitenko 1994), while ducks develop HCC following treatment with levels as low as 0.02 mg/kg/month over a 2 year period (Cova et al. 1990). A similar trend is observed in mouse and rat sensitivity. One of the most important findings among variations in species susceptibility has been delineated in the differences in conjugation of the carcinogenic AFB_1-8,9-epoxide with glutathione (GSH) in rat and mouse models (Monroe and Eaton 1987; O'Brien et al. 1983; Quinn et al. 1990). Mice are relatively insensitive to induction of tumors in the liver following AFB_1 treatment, even though epoxidation of AFB_1 is high compared to rats. A high level of glutathione S-transferase (GST) activity has been reported to be important for species resistance to AFB_1 (Eaton et al. 1994). Specific GST activity for AFB_1-8,9-epoxide conjugation by homologous rat and mouse forms was 100-times higher in the mouse than the rat (Eaton et al. 1994).

These differences in metabolism are likely to result in the over five-times higher susceptibility observed in the rat.

The carcinogenic potency of the AFs is highest for the AFB_1 congener. Development of lesions in the liver has been thoroughly studied in multiple animal species; however rat and trout models have been the most frequently used. The first study to demonstrate effects of chronic AF exposure in a laboratory animal was conducted by Wogan et al. (1974). Rats fed levels of 1, 5, 15, 50, and 100 ng/g AF developed tumors at incidences of 9, 4.5, 19, 80, and 100% (Kensler et al. 2011; Wogan et al. 1974). This work indicated that HCC could develop at doses as low as micrograms per day, implicating the carcinogenic potential of AF consumption in human populations. Extrapolation of animal model carcinogenicity to risk assessment in human populations has been most useful in various studies with the monkey and rainbow trout, whose sensitivity is most closely related to humans. In a model utilizing three separate species of monkeys, (rhesus, cynomolgus, and African green) liver tumors developed in 64% of animals treated with AFB_1 for 6 months when doses were steadily increased from 0.2 to 0.8 mg/kg (Thorgeirsson et al. 1994). Studies in rats and rainbow trout exhibit a dose-response relationship between AFB_1 exposure levels and DNA adduct formation even at levels relative to chronic human exposures (Buss et al. 1990; Dashwood et al. 1988). Therefore, it is widely accepted that AFB_1 has no threshold level at which it would not pose a genotoxic risk.

Carcinogenicity of AFB_1 is dependent on activation through biological metabolism to the AFB_1-8,9-epoxide. Following ingestion and absorption, AFB_1 is

biotransformed predominantly by hepatocytes by both phase I and phase II metabolic enzymes. The AFB_1-8,9-epoxide can react with biological nucleophiles, such as DNA, RNA, and proteins causing disruption of normal cellular function and DNA mutations. AFB_1 is considered a complete carcinogen due to its ability to both induce and promote cancer development ((IARC) 1993, 2002). The ability of AFB_1 to act as an initiator stems from its bioactivation to a direct-acting mutagen and subsequent disruption of several genes involved in carcinogenesis. The AFB_1-8,9-epoxide reacts with guanine at the N-7 position resulting in an AFB_1-DNA adduct and G:C→T:A transversions. This adduct, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N^7-Gua), is the most abundant occurring in animal models (Croy et al. 1978; Croy and Wogan 1981a, b; Essigmann et al. 1977; Lin JK et al. 1977). The importance of guanine as a target for AFB_1 genotoxicity has been further solidified through various carcinogenicity studies showing G:C to T:A or G:C to A:T point mutations in AFB_1 induced tumors (McMahon et al. 1987; McMahon et al. 1986; Sinha et al. 1988). These transversions were all observed at codon 12 of the K-ras gene. K-ras, a protooncogene, has been suggested as a target gene for AFB_1 induced point mutations of the DNA. Evidence from a rat study conducted by Soman and Wogan (1993) showed point mutations in all AF related adenomas and carcinomas at the K-ras gene. High frequency of mutations at codon 249 of the p53 tumor suppressor gene in human HCC cases has been reported in areas with chronic AF exposure (Bressac et al. 1991; Hsu et al. 1991). However, these studies were conducted in populations that are also afflicted with endemic HBV infection and therefore the observed mutations could not be solely attributed to AF. Multiple animal

models have failed to support the evidence of selective p53 mutations, on a codon 249 equivalent, from AFB_1-induced carcinogenicity (Wild and Montesano 2009). Human liver epithelial cells that were transfected with HBx gene and treated with AFB_1 were more sensitive to the cytotoxicity of AFB_1 and were sensitive to induction of mutations at codon 249 of p53 (Sohn et al. 2000). Thus, it is speculated that the codon 249 hotspot mutations observed in epidemiological studies is dependent on having both active HBV surface antigen and AFB_1 present (Wild and Montesano 2009).

The International Agency for Research on Cancer (IARC) has classified AFB_1 as a Group 1 human carcinogen based on multiple epidemiological studies in populations with high HCC incidence ((IARC) 1993, 2002). Heterogeneity of AF contamination in crops and food-stuffs, differences in food intake, and metabolism between individuals makes exposure assessment difficult through analysis of food samples. Thus, use of biological metabolites resulting from AFB_1 exposure, termed biomarkers, are the current standard for evaluating exposure in human populations and subsequent correlations with endpoints of disease. A thorough review of AFB_1 biomarkers of exposure is discussed in subsequent sections; here biomarkers will only be referred to in the context of correlating human AFB_1 exposure with HCC. Some of the first work to determine associations of AFB_1 consumption with HCC development in humans involved case-control studies in Mozambique, Swaziland, and the Philippines (Bulatao-Jayme et al. 1982; Linsell and Peers 1977; Peers and Linsell 1977; Van Rensburg et al. 1985). These studies estimated dietary intake of AFs, retrospectively, for HCC cases and compared them with age and gender matched controls. Results from all three studies indicated that

mean AF exposure was positively correlated with HCC, with daily AF exposure in HCC cases estimated to be 4.5 times higher than in controls. Similarly, in Taiwan, detectable levels of AFB_1 biomarkers were a risk factor for HCC, with an odds ratio of 1.5 compared to controls (Lunn et al. 1997). The population of Qidong, China has been investigated over numerous years for AF, hepatitis B viral infection (HBV), and HCC incidence. This particular population has been a keystone in discovering effects of chronic AF exposure in humans, due to the seemingly inextricable contamination of foods and endemic HBV and HCC incidence. Sun et al. (1999) concluded that the attributed relative risk for HCC from AFB_1 was 0.55 and thus, AF exposure accounted for a substantial portion of HCC cases in the screened population even in the presence of HBV (Sun Z et al. 1999). Individuals with HBV positive surface antigen (HBsAg) had a 10-fold increased rate of HCC, when they also were consuming large amounts of AF compared to those with positive HBsAg and low exposure levels to AFs (Yeh et al. 1985, 1989). Importantly, populations that are most at risk for AF exposure are often also populations with endemic HBV infection. Therefore, the epidemiology of AF induced HCC often cannot be considered without also accounting for chronic HBV infection.

Like AFB_1, chronic hepatitis infection has been categorized by IARC as hepatocarcinogenic in humans (IARC 1994). It is estimated that there are 400 million chronic HBV carriers worldwide (Parkin et al. 2005). DNA insertion by HBV under chronic infection conditions would result in genomic instability and ultimately liver disease and/or HCC. Also, chronic inflammation and generation of reactive oxygen

species resulting from HBV infection could have promoter-like activity, thereby increasing the incidence of AFB_1 induced cell proliferation leading to tumor development. Multiple epidemiological studies have investigated the effect of the complex paradigm of chronic AF exposure and HBV infection on HCC development. It has been shown that the presence of HBV infection coupled with AFB_1 exposure increases the risk of hepatocellular carcinoma (HCC) by up to 3.5-fold when compared to those who are seropositive for HBV in the absence of AFB_1 exposure (Ming et al. 2002; Sun Z et al. 1999). The activities of HBV and AF biomarkers as independent and interactive variables for risk of HCC were studied in a cohort of 18,244 men in Shanghai (Qian GS et al. 1994; Ross et al. 1992b). This duo of studies showed increases of HCC by, 3.4-fold when AF biomarkers were detected in the urine alone, 7.3-fold when participants tested positive for HBsAg alone, and 59.4-fold when participants tested positive for both HBsAg and AF biomarkers, respectively. These results and other studies have clearly established the synergistic interaction between AFB_1 and HBV that occurs in the development of HCC. Wang LY et al. (1996) confirmed these findings in Taiwan, where HCC incidence in HBV carriers with detectable AF biomarkers had an odds ratio of 2.8 compared to those with non-detectable biomarkers. Similarly, when participants were classified as either having high or low AFB_1 exposure, the odds ratio (5.5) clearly suggested that high exposure was a risk factor in HCC cases. HCC risk in HBsAg positive participants revealed a dose-dependent relationship with urinary AF biomarkers (Yu et al. 1997); providing further evidence of a causal relationship between AF exposure and HCC risk in human populations in both the presence and absence of

chronic HBV. Importantly, a recent risk assessment for global burden of AF-induced HCC attributed from 25,200 to 155,000 of the 550,000 annual cases of HCC to AF exposure alone (Liu Y and Wu 2010).

1.1.5 Nutritional modulation

Although the vast majority of AF based research focuses on the genotoxicity and carcinogenic properties of AFB_1 the first sign of exposure in all animal species is decreased growth and loss of appetite. This observation has led to the investigation of the role AF has in growth faltering and nutritional status. Food conversion efficiency is consistently decreased in animals exposed to AF versus those on control diets. Following administration of AF to broiler chicks and barrows, vitamin A levels were decreased in serum and liver (Harvey et al. 1994; Pimpukdee et al. 2004). AF-contaminated corn (500 ng/g AFB_1) also decreased serum vitamins A and E by half in young pigs exposed for 21 days (Harper et al. 2010). Alternatively, vitamin E levels significantly increased as AF levels decreased in a Ghanaian clinical trial designed to mitigate exposure (Afriyie-Gyawu et al. 2008b), and vitamin A levels were not correlated with AF-alb biomarkers in another human trial (Turner et al. 2003). These findings were contradictory to a study conducted in 2010 in Ghana, where individuals with high AF-alb levels had a 2.64-fold greater risk of vitamin A deficiency compared with those people with low AF-alb (Obuseh et al. 2010). A similar trend was observed for vitamin E deficiency; however it was not statistically significant. These differences could be a result of study design, population size, and/or HBV infection. For example, Obuseh et al., (2010) utilized a cross-sectional design with 147 adult participants; however Turner et al. (2003)

conducted a cohort study with 472 children ages 6-9. It is also important to note that high AF-alb levels were associated with an increased relative risk (odds ratio, 5.88) for HBsAg (Obuseh et al. 2010). The other two studies conducted in humans did not report HBV infection. Interestingly, Pan et al. (1993) observed an inverse correlation between serum vitamin A levels and development of HCC in HBsAg positive participants. Therefore, it is a possibility that HBV infection may play a role in lowered vitamin A levels in these human populations along with AF exposure. Vitamin A may also have a protective effect on exposure to carcinogens, like AF, by inhibiting their metabolic activation and preventing the initial steps of carcinogenesis (Huang et al. 1982). Thus, more research needs to be conducted to investigate the observed differences in serum vitamins A and E. Although AF-alb levels appear to be associated with vitamin A and vitamin E levels it is still unclear if this correlation is a result of changes in AFB_1 metabolism via vitamin A mediated mechanisms or if AF exposure directly affects vitamin absorption and/or metabolism.

Vitamin D, zinc, and selenium were affected by AF exposure in animal models; however reports of these effects are limited. Broiler chickens had decreased vitamin D levels following treatment with 1 ppm AF for 5 days (Glahn et al. 1991). Aflatoxicosis studies in chickens demonstrated an inverse correlation between AF exposure and serum selenium levels (Hegazy and Adachi 2000). Chen et al. (2000) also observed a negative correlation between selenium and AF-alb levels from a Chinese population. Maternal AF exposure in pigs resulted in decreased zinc content in offspring, although there were no significant changes in the maternal zinc concentrations (Mocchegiani et al. 2001).

Lowered zinc in the piglets resulted in decreased cellular immunity due to lowered thymulin activity. Reduced zinc concentrations following AF exposure has also been observed in rats (Doyle et al. 1977; Ikegwuonu 1984). Influences of AF on all of these nutrients could have negative impacts on the immune system and growth. Other reports indicate that AF exposure can lead to obstructed biliary flow (Neeff et al. 2013; Wouters et al. 2013), in conjunction with dose-dependent increases of bilirubin and alkaline phosphatase in rats (Clifford and Rees 1967), goats (Clark et al. 1984), monkeys (Rao and Gehring 1971), and pigs (Cardeilhac et al. 1970). Results from these studies suggest that fat-soluble vitamins may not be fully absorbed from food due to decreased bile release from the liver. Recently, Gong YY et al. (2012) reported that, after adjusting for parasitic infection, AF exposure significantly contributed to the incidence of hepatomegaly in Kenyan school children, a condition which can be caused by a variety of ailments in children including biliary obstruction and anemia (Wolf and Lavine 2000). In addition to hepatic damage, AF also causes toxicity of the intestinal mucosa, resulting in decreased nutrient uptake (Applegate et al. 2009; Yunus et al. 2011a; Yunus et al. 2011b). Although the concept of AF acting as an anti-nutrient is commonly accepted among mycotoxin researchers, the mechanisms behind these effects are still not well established and consequently cannot be applied to risk assessment for human populations.

1.1.6 Growth suppression

AF exposure has been strongly associated with growth impairment in various animal species (Cheng et al. 2001; Giambrone et al. 1985; Harvey et al. 1995, 1989a,

1989b; Kocabas et al. 2003; Pimpukdee et al. 2004). Overall, research has not only indicated reduced feed intake and lowered weight gain in mice, chickens, pigs, ducks, and turkeys, but has also shown that AF exposure correlates with lower feed conversion efficiency (Khlangwiset et al. 2011). In 30 animal studies designed to assess deleterious effects of AF on growth, 29 out of 30 reported that exposed animals exhibited either reduced weight gain or decreased feed conversion efficiency. Furthermore, multiple studies investigating the effects of AF on *in utero* development have reported reduced fetal weight and/or length (Khlangwiset et al. 2011). Turner et al. (2007) followed the growth of 138 Gambian infants from birth to 12 months and compared growth status with maternal AF exposure during pregnancy. In this study, a higher mean maternal AF-alb concentration was significantly correlated with lower weight-for-age and height-for-age z-scores. Maternal cord blood and infant blood samples were also assessed for AF-alb, yielding detectable biomarker levels in 48.5% and 11% of samples, respectively. Furthermore, children ages 16-37 months from Benin exhibited a significant negative correlation between AF exposure (AF-alb) and height increase over 8-months (Gong YY et al. 2004). The growth stunting observed in children from Benin and Togo had a distinct dose-response relationship with AF-alb levels in the serum. A height-for-age z-score of $\leq$ -2 had 30-40% higher AF-alb when compared to children not classified as stunted (Gong YY et al. 2002, 2003). Relationships observed between AF exposure and impaired growth are hypothesized to work through a complex mechanism that results in impaired gut health and function, decreased nutrient absorption, and chronic inflammation (Smith LE et al. 2012). Definition of the threshold for effect on growth,

nutritional health, and immunology is important in the effort to provide effective intervention strategies to reduce health consequences from AF exposure in developing countries.

1.1.7 Immune suppression

Nutritional interference resulting from AF exposure can have further influences on the immune status and overall health in those chronically exposed. AFs have been implicated as immune-toxicants in multiple animal species. Research has demonstrated deleterious effects on the innate immunity, such as phagocytic cell function following AF treatment in laboratory and domestic animals. Negative effects on human immunity are still inconclusive and more research needs to be conducted to make conclusions about possible health effects in high-risk populations. Communities that are frequently exposed to AFs are often also highly exposed to infectious pathogens. Low dose levels of AFB_1 have diminished delayed-type of hypersensitivity, which is an important aspect of cell-mediated immunity. Both T and B cell responses have been reported to be lowered following AFB_1 treatment. However, the findings from AFB_1 related immune studies should be interpreted carefully as results have proven inconsistent. Different models, doses, and exposure duration seem to add variability to results, leading to some conclusions that AFB_1 stimulates the immune response while others indicate a suppression of immunity. However, there is significant evidence in multiple domestic animal species that AFB_1 suppresses T-cell mediated immunity, particularly in cattle (Bodine et al. 1984; Brown RW et al. 1981), poultry (Ghosh et al. 1990; Giambrone et al. 1985), and swine (Liu BH et al. 2002; Mocchegiani et al. 1998). A recent study in

rats following a short (i.e. one-week) or long exposure (i.e. five-weeks) to AFB_1 has concluded that short-term exposures may act to suppress cell-mediated immunity while the long-term exposure may act to enhance cytokine excretion and an increase in inflammation and apoptosis (Qian G et al. 2013a).

Following the one-week dose period, 25 μg AFB_1/kg body weight decreased cytotoxic T cells ($CD8^+$), and $CD3^-CD8a^+$ natural killer (NK) cells. Expression of interleukin (IL)-4 and interferon (IFN)-ɣ by $CD4^+$ and $CD8a^+$ T cells was inhibited by AFB_1 treatment. However, many of these effects were differential following the five-week dose period, depending on AFB_1 dose. IL-4 expression by CD4+ T cells was consistently decreased with one and five weeks of treatment. IFN-ɣ and tumor necrosis factor (TNF)-α expression was elevated following five weeks of AFB_1 treatment and this is in contradiction to the inhibition observed after one week of AFB_1 (Qian G et al. 2013a). The authors of this study concluded that short-term exposure to AFB_1 can dose-dependently lower the number of splenic $CD8^+$ lymphocytes and NK cells and subsequent production of cytokines by $CD4^+$, $CD8^+$, and NK cells. Inversely, long-term exposure can result in stimulatory effects on such lymphocytes and expression of IFN-ɣ and TNF-α at low dose levels. IL-4 contains anti-inflammatory properties while IFN-ɣ and TNF-α are pro-inflammatory (Niiro et al. 1997). This would suggest that chronic and sub-chronic exposure to AFB_1 could play a role in the establishment of a chronic inflammatory environment as a result of cytokine excretion. Similarly, Bruneau et al. (2012) showed, *in vitro*, that murine macrophages would decrease expression of anti-inflammatory cytokine IL-10, but increase in pro-inflammatory IL-6 following pre-

treatment with AF for 72 hr. AFB_1 exposure reduced helper T cells in the spleen and antibody response in mice (Hatori et al. 1991), inhibited excretion of IL-2, IL-3 and IFN-ɣ by macrophages (Dugyala and Sharma 1996), and decreased murine macrophage phagocytosis and production of TNF-α in multiple cellular and animal models (Moon et al. 1999a, b). Dietary exposure to AFB_1 in rats at levels from 0.01-1.6 mg/kg inhibited production of IL-1, IL-2, and IL-6 as well as increased the percentages of T cells but lowered B cells following 12 weeks of treatment (Hinton et al. 2003).

Human populations that are at high-risk for AFB_1 exposure are also likely to have frequent insults to their immune system from infectious pathogens or bacterial disease and therefore, it is difficult to ascertain effects of AF on human immunity. One human study in Ghana assessed correlations between AF-alb serum biomarkers and percentages of leukocyte immunophenotypes in peripheral blood (Jiang et al. 2005). Participants in this study with high levels of AF-alb had significantly lower levels of $CD3^+$ and $CD19^+$ cells, with activation marker $CD69^+$, than those people with low AF-alb. $CD8^+$ T cells were also significantly lower in participants classified in the high AFB_1 exposure group. Implications for the effects immunomodulation could have on such populations are demonstrated in a report on immune dysfunction in AFB_1 exposed individuals infected with human immunodeficiency virus (HIV). Results from this study indicated that high AFB_1 consumption may heighten some HIV associated changes in T-cell and B-cell phenotypes, possibly accelerating the development of acquired immunodeficiency syndrome (AIDS) (Jiang et al. 2008). A cohort of children from Gambia showed a negative correlation in AF-alb levels and secretory immunoglobulin A

(IgA) from the saliva (Turner et al. 2003). IgA in mucosal barriers acts as a barrier for mucosal immunity to protect the body from potential infections. In populations frequently exposed to infectious insults an AFB_1-mediated decrease in IgA could influence one's susceptibility to infection.

Importantly, lymphoid cells such as cytotoxic T-cells, NK cells, and other T-lymphocytes function to directly or indirectly kill tumor cells. Therefore the inhibitory effects of AFB_1 on such cells could have a pronounced effect not only on overall immunity, but also on tumor progression (Raisuddin et al. 1991). Effects on the immune system from AFB_1 could also affect cancer progression through the development of chronic inflammation, which can stimulate cellular mitosis and promotion of initiated cancer cells. The complex dichotomous relationship of AFB_1 with immune suppression and/or increased inflammatory response has serious implications for human health in populations that are frequently exposed to infectious diseases or at risk for carcinoma.

1.1.8 Human exposure in Africa

AF exposure is most common in Southeast Asia, China, Africa, and the Caribbean. Human exposure most commonly occurs through frequent consumption of staple foods containing low levels of AFs. With global changes in climate over the past century it has been hypothesized that populations living between 40° N and S latitude are at risk for AF exposure, which includes the southern half of the United States (Williams et al. 2004). However, the United States Food and Drug Administration (FDA) regulates levels of AFs in commercially available foods. Populations living in agricultural or low income communities are at higher risk of chronic exposure due to food instability, which

results in the consumption of a low-grade, homogenous diet. Crops produced and stored in tropical and sub-tropical regions are vulnerable for contamination, where humidity and drought are common and conducive for *Aspergillus* growth (Williams et al. 2004). Historically, acute aflatoxicosis in humans has resulted in multiple deaths. Two different reports from India, during 1974, indicated that the deaths of 106 and 97 people were associated with consumption of contaminated maize (Krishnamachari et al. 1975; Tandon et al. 1977). Africa, in particular, has a high incidence of AF exposure and continues to report multiple clusters of aflatoxicosis cases in humans. In 2004, a severe outbreak occurred in Kenya where 317 clinical cases were registered and 125 people died after consumption of maize contaminated with AF at levels as high as 1000 ppm (CDC and Prevention 2004). In October of 2011, the Kenya Red Cross recalled 360 tons of contaminated relief foods containing sub-lethal levels of AFs. It was estimated that 60,000 children were put at risk after ingesting the contaminated food before the recall occurred (allAfrica 2011).

Crop contamination and subsequent consumption of AF is commonly observed in staple cereal grains, such as, maize, rice, wheat, and groundnuts, such as peanuts (CAST 2003). These crops are typically a primary food source in developing countries, resulting in high-risk for both acute and chronic exposure in much of the population in developing countries. African populations in particular consume some of the highest amounts of maize and peanuts per person per day (Liu Y and Wu 2010). Studies utilizing biomarkers of exposure in West Africa have indicated on-going, extensive exposure (Egal et al. 2005; Njumbe Ediage et al. 2013; Shephard 2008; Wild et al. 1992;

Zarba et al. 1992). Exposure in a particular community, in the Ashanti Region of Ghana, has been well documented for numerous years, indicating chronic exposure over the past 10 years (Jolly et al. 2006; Kumi et al. 2011; Mitchell NJ et al. 2013; Wang P et al. 2008). AF exposure in this community, like others in Western Africa, can occur in humans *in utero* and continue through life. Detectable levels of AFs have been observed in umbilical cord blood samples taken at birth in populations where mothers are highly exposed (De Vries et al. 1989; Lamplugh et al. 1988; Turner et al. 2007). Importantly, a recent study conducted to assess the transfer and metabolism of AFB_1 in human placentas was undertaken in Finland (Partanen et al. 2010). Researchers conducted perfusions of human placenta samples immediately following birth and provided the first direct evidence of the actual transfer of AFB_1 across the placenta and into the fetal circulation. Based on this research the sole AFB_1 metabolite produced by xenobiotic enzymes present in the placenta is thought to be aflatoxicol (AFL). AFs have also been detected in human breast milk (Adejumo et al. 2013; Coulter et al. 1984; Elzupir et al. 2012; Jonsyn et al. 1995; Lamplugh et al. 1988; Wild et al. 1987; Zarba et al. 1992) and weaning foods (Gong YY et al. 2003; Gong YY et al. 2004; Kumi et al. 2011; Oyelami et al. 1996). Estimated exposure of adults in African populations ranges from 3.5 to 850 ng/kg body weight/day (Liu Y and Wu 2010; Shephard 2008). Some of the highest exposure estimates in Africa are reported by Shephard (2008). During this risk assessment it was proposed that the highest exposure in Africa occurs in Ghana due to the large consumption of maize. Based on the assumption of an average 60 kg adult eating 1000 g/day of maize, exposure could be as high as 850 ng/kg body weight/day;

this accounts for a population risk of 70 liver cancer cases per 100,000 people/year in Ghana (Shephard 2008). Similarly, Liu and Wu have estimated that HCC cases attributable to AF consumption is highest in Africa accounting for 40% of those worldwide, with 2,150 to 9,300 annual incidences in Africa (Liu Y and Wu 2010). However, it is important to note that these extrapolations are typically based on levels of contamination in samples of food-stuffs and averages of total food consumption per day. In applying such evaluations of exposure to human risk assessment there are certain factors that should be recognized. First, levels of *Aspergillus* and subsequently AF contamination are temporally variable and typically result in heterogeneous or "hot spot" confluence of food-stuffs. Although analysis of AF levels in food is a critical tool for exposure assessment, it should be acknowledged that the values reported from these methods have the potential to grossly over or under estimate true exposure in the population of interest.

AF associated health burdens in Africa are considerably higher once growth faltering, suppressed immunity, and incidences of acute aflatoxicosis are taken into account. Multiple factors contribute to the etiology of AF related disease outcomes in Africa. For example, AF exposure has been inversely correlated with various socioeconomic factors, such as income, education level, employment, and number of dependents in a household (Adejumo et al. 2013; Jolly et al. 2006; Shuaib et al. 2012). All of these factors could negatively influence the diversity of the diet and the quality of food consumed. However, it is important to note that there are reports of such socioeconomic statuses having no influence on AF exposure in Kenya and Malaysia

(Leong et al. 2012; Yard et al. 2013). A lack of effect observed in these studies could indicate that AF contamination is universally prevalent in the food supply and thus, effects health across all economic classes. Other contributors to the etiology of AF exposure are climate and cultural practices. As stated previously, Aspergillus growth and production of toxic AF metabolites is heavily influenced by drought, insect damage, and humidity. Sub-Saharan Africa is afflicted with cases of severe drought and famine. Drought negatively effects plant health and ability to stave off insect and fungal infestation. Although drought is common in sub-Saharan Africa, there is also high humidity content, which can create an optimal environment for fungal growth during storage of crops. Many storage and eating habits are culturally based. For instance, many African communities eat a high quantity of fermented maize. The traditional fermentation process in Ghana, in particular, occurs without addition of an accelerant. Typically maize is mixed with water until moist and allowed to ferment naturally for three days. The average household will make enough of this fermented dough to supply the family with meals for a week and store at room temperature until consumed. This practice results in growth of various fungi on the dough, which is desirable for taste and textural preferences. These types of cultural practices could potentiate AF contamination and are difficult if not impossible to change.

1.1.9 AFB_1 metabolism

Approximately 50% of an orally administered dose of AFB_1 is absorbed from the duodenal region of the small intestine (Hsieh and Wong 1994). Although there is a lack of research published on the mechanisms of AF absorption, one study indicated that the

uptake of AFB_1 in the intestine occurs through passive diffusion (Kumagai 1989). The authors made this conclusion following the observation that the rate of AFB_1 absorption increases nearly in proportion to an increase in AFB_1 concentration. Interestingly, this study also indicated that the rate of absorption of AF was dependent on the lipophilicity of the compound. The rate of AFG_1 absorption, a less lipophilic compound, was significantly lower than the rate for AFB_1 following perfusion of the duodenum (Kumagai 1989). A recent in vitro study with human intestinal Caco-2 cells seems to support passive diffusion as the mechanism for AF absorption (Caloni et al. 2012). The uptake and efflux ratios of an AFB_1 metabolite present in breast milk (AFM_1) were <2, suggesting that AFM_1 was passively transported. Furthermore, intracellular concentrations of AFM_1 were very low and not dose dependent, indicating that Caco-2 cells do not actively absorb AFM_1.

Following absorption to the vasculature, the majority of AF is concentrated in the liver, where AFB_1 biotransformation by phase I and II enzymes results in the production of both activated and detoxified metabolites. Oxidation of AFB_1 by Cytochrome P450 enzymes (CYPs) is the primary mechanism of phase I AF metabolism. Epoxidation of the double bond on the terminal furan ring of AFB_1 and AFG_1 produces a genotoxic metabolite that can alkylate with nucleic acids (Eaton and Gallagher 1994; Essigmann et al. 1977; Swenson et al. 1977). Human CYPs: 1A2, 2A3, 2B7, 3A3, and 3A4 have all been implicated in the oxidative metabolism of AFB_1 (Massey et al. 1995). CYP1A2 and 3A4 are the predominant phase I enzymes that facilitate formation of the carcinogenic AFB_1 exo-8,9-epoxide metabolite. When compared to CYP3A4 activity, CYP1A2 was

found to have a higher-affinity for AFB_1 activation at low substrate concentrations relevant to human exposures (Gallagher et al. 1994). However, liver samples from Thailand showed a stronger correlation with CYP3A4 expression and AFB_1 epoxidation at intermediate AFB_1 concentrations relative to correlations with CYP1A2 expression (Kirby et al. 1993). Furthermore, it appears that CYP3A4 is constitutively expressed at higher levels than CYP1A2 in the human liver and could thus be the primary enzyme for AFB_1 activation in humans (Gallagher et al. 1994; Kirby et al. 1993). The AFB_1-epoxide is highly unstable and capable of covalently binding with DNA, resulting in irreparable adducts at the N-7 guanine position (Essigmann et al. 1977; Lin JK et al. 1977; Martin and Garner 1977).

In addition to epoxidation of AFB_1, phase I metabolism includes hydroxylation and demethylation, forming the M_1 (AFM_1), Q_1 (AFQ_1), P_1 (AFP_1), $B2_a$ ($AFB2_a$), and aflatoxicol metabolites. These products exhibit less than 4% of the mutagenic activity of AFB_1 in Salmonella assays (Coulombe et al. 1982; Essigmann et al. 1982; Gurtoo et al. 1978; Wong and Hsieh 1976), therefore metabolism to the hydroxylated and demethylated metabolites is considered a detoxification process. Hydroxylation of AFB_1 to AFQ_1 is increased following treatment with glucocorticoid-type inducers, implicating CYP3A subtypes as the active enzymes in formation of AFQ_1 (Halvorson et al. 1988; Raney et al. 1992b). In contrast, AFM_1 formation is catalyzed by the CYP1A subfamily of P450 enzymes (Koser et al. 1988; Metcalfe et al. 1981; Raina et al. 1985; Santhanam et al. 1989). An interesting epidemiological finding in a human population with high incidence of HCC and AF exposure in the Fujian Province of China further supported

the CYP1A mediated detoxification of AFB_1 to AFM_1 (Lin L et al. 1991). A higher incidence of hepatomas was found in participants who were nonsmokers compared to smokers over the age of 50 (odds ratio 2.06). It was hypothesized that the polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke could induce CYP1A enzymes, leading to increased formation of AFM_1, pushing AFB_1 metabolism into the detoxification pathway.

Phase II metabolism further transforms the phase I metabolites to higher molecular weight products for excretion. Conjugation of the epoxide with glutathione (GSH) and the hydroxylated metabolites with glucaronic acid are the major metabolites produced from phase II detoxification. Production of an AFB_1-8,9-epoxide-GSH conjugate is catalyzed by glutathione S-transferases (GSTs) and has been identified as the primary metabolite in the bile following AFB_1 treatment (Degen and Neumann 1978; Holeski et al. 1987; Raney et al. 1992a). Conjugation with GSH is typically followed by conversion to an AF-mercapturic acid residue by acetylases and peptidases; this metabolite is then excreted in the urine (Wild and Turner 2002). As previously discussed in section 1.3.1, GSH conjugation and GST activity are important in the susceptibility of different species to AFB_1 toxicity. For instance, oral doses as high as 10,000 ppb AFB_1 do not cause liver cancer in mice while levels as low as 15 ppb cause increased tumors in rats (Wogan and Newberne 1967). This difference in susceptibility can be explained by the fact that mice constitutively express murine GST A3-3, which catalyzes conjugation to 50 times faster than in sensitive species (Eaton and Gallagher 1994). Humans are thought to have a metabolic capacity similar to that of the rat with regard to AFB_1

detoxification. Human GSTs do not readily conjugate the epoxide metabolite, resulting in a longer half-life and increased exposure *in vivo*. Glucuronidation of AFM_1, AFP_1, and aflatoxicol are important steps that enhance their rates of elimination from the body. Conjugation with glucuronide appears to occur at a higher rate with AFP_1 compared to the other hydroxylated metabolites (Eaton et al. 1994). Many of the AFB_1 metabolites are utilized in human studies as biomarkers to assess exposure and effect (Figure 2).

1.1.10 Biomarkers of exposure

As previously indicated, mycotoxin exposure assessment in human populations is difficult through food screening due to the heterogeneous nature of mycotoxin contamination and human dietary habits. Similarly, predictions and associations with AF consumption and health outcomes are difficult to assess in humans due to the latent nature of AF-induced disease. Therefore an effort to discover biomarkers that correlate with xenobiotic ingestion was initiated over 20 years ago for use in human based epidemiological and chemoprevention trials (Turner et al. 2012; Wild and Turner 2001, 2002). Primary metabolites excreted in the urine, such as AFM_1, AFP_1, and AFQ_1, were some of the first metabolites investigated as potential biomarkers of exposure in diverse human populations (Gan et al. 1988; Groopman et al. 1992c; Ross et al. 1992a, 1992b; Zhu et al. 1987). During a cross-sectional survey in Zimbabwe, AFM_1 was found in the urine at a higher rate than any other metabolite from 1200 human samples (Nyathi et al. 1987). Following this report Zhu et al. (1987) demonstrated that urinary AFM_1 excretion was also linearly related to AFB_1 consumption. This study collected daily urine samples and food samples from 32 households in Fushui County of the Guangxi Autonomous

Region of the People's Republic of China and analyzed each for AFM_1 and AFB_1, respectively. Results indicated a strong positive correlation (correlation coefficient of 0.66) between dietary AFB_1 intake and urinary AFM_1 excretion (Zhu et al. 1987). Dosimetry analysis in a rat model confirmed the linear relationship between AFM_1 in the urine and AFB_1 intake with an even stronger correlation coefficient of 0.93 (Riley et al. 1993). Data from this study and Groopman et al. (1992c) estimated that between 1.2 and 2.2% of the total AFB_1 intake is excreted as AFM_1 in the urine. Excretion of AFM_1 occurs rapidly, following an oral dose of AFB_1, typically peaking in the first 12 hr and decreasing to undetectable levels within 48 hr (Dalezios et al. 1973; Groopman et al. 1988; Mitchell NJ et al. 2013; Sarr et al. 1995). Thus, level of urinary AFM_1 is indicative of AF exposure within the past two days. Interestingly, AFM_1 received its name due to its presence and discovery in breast milk from AF exposed mothers. Although this marker can be used to determine mother-child exposure, it is not commonly used in exposure assessment for adults. Total excretion of AFM_1 in breast milk has been estimated to account for only 0.09 to 0.43% of AFB_1 intake of the mother (Zarba et al. 1992). Urinary AFP_1 and AFB_1 are not linearly correlated with AF intake in humans or rats (Groopman et al. 1992a, 1992b. 1992c).

Scholl PF et al. (1997) investigated the use of AFB_1-mercapturic acid as a biomarker of carcinogenic susceptibility in rats. Because the AFB_1-mercapturic acid is a product of the GST mediated detoxification pathway for the AFB_1 epoxide, it was speculated that this marker could be utilized in intervention therapy trials intended to induce the rate of GST activity. Although there was a dose-dependent relationship

between AF treatment and excretion of mercapturic acid conjugates, this biomarker has not been extensively utilized.

AFM_1 is an important tool for assessing exposure in human populations; however, AFB-N^7-guanine excretion in the urine is a useful biomarker of effect. AFB-N^7-guanine is the primary AFB_1-DNA adduct excised from the DNA and present in urine (Croy et al. 1978; Essigmann et al. 1977). This biomarker is removed rapidly from the DNA and has a half-life of 8-10 hr in rats (Bennett et al. 1981; Groopman et al. 1980). A correlation coefficient of 0.99 was found in rats indicating a strong relationship between a p.o. dose of AFB_1 and urinary concentration of the genotoxic metabolite (Groopman et al. 1992b). This correlation was also observed in a genetically diverse human population from China (correlation coefficient: 0.65) (Groopman et al. 1992c). One of the most striking studies that provides evidence for AFB-N^7-guanine use as a biomarker of biological effect was determined in a prospective study, which is the most rigorous test for association between a suspected causal agent and disease outcome. Ross et al. (1992b) conducted a prospective study in Shanghai with 18,244 male participants starting in 1986 and concluding three and a half years later. Of the study participants, 22 developed HCC within the study duration. Analysis of urine samples indicated that the presence of AF biomarkers carried a relative risk of 3.8 for development of liver cancer. AFB-N^7-guanine in particular showed a highly significant increase in relative risk (RR= 4.9) for cancer development (Ross et al. 1992b). Interestingly, the relative risk for liver cancer in participants with positive AF markers and HBsAg was 60. This study was the first to show both a synergistic interaction between HBsAg and AF for liver cancer

development, and a positive relationship between an AFB_1 genotoxic metabolite and disease outcome.

Although the use of urinary biomarkers such as AFM_1 and AFB-N^7-guanine have been useful in epidemiology based research, their short half-lives make exposure assessment a moving target due to high variability in AF intake and subsequent excretion. Thus, studies utilizing these markers require large population sizes and multiple samplings for strength of data analysis. The AFB_1-albumin adduct (AFB_1-alb) however has a half-life equivalent to that of serum albumin (Sabbioni et al. 1987), which is 20 days in humans and 2.6 days in rats (Peters 1970; Schreiber et al. 1971). During AFB_1 metabolism, the epoxide can be hydrolyzed spontaneously in the presence of water to produce an 8,9-dihydrodiol which is reactive with proteins and binds to one or more lysine residues in serum albumin, which can be quantified in animals and humans (Sabbioni et al. 1990; Sabbioni et al. 1987; Skipper et al. 1985; Wild et al. 1986). The extant scientific literature labels this biomarker as either AFB_1-albumin (AFB_1-alb) or AFB_1-lysine depending on the context; here AFB_1-alb will be used in reference to this metabolite. Molecular dosimetry of AFB_1-alb and AF intake in humans was first described by Gan et al. in 1988. During this study linear regression models demonstrated a strong positive correlation between the biomarker and exposure (Gan et al. 1988). Data from this study also indicated that the AFB_1-alb adduct levels represent approximately 1.4 to 2.3% of a daily dose of AFB_1. This is similar to rat (1 to 2%) (Sabbioni et al. 1987; Wild et al. 1986) indicating the rat as an appropriate model for human excretion. Due to the 20 day turnover rate in humans for albumin it has been estimated that AFB_1-

alb levels are 30 times higher than that produced by a one-day exposure and can reach a steady state in chronically exposed populations (Gan et al. 1988; Groopman et al. 1994; Sabbioni et al. 1987). AFM_1, AFB_1-alb, and AFB-N^7-guanine have been integral to epidemiological surveys and risk assessment as well as determining efficacy in intervention trials in human populations.

1.1.11 Regulation

Governmental regulations to reduce AF exposure in animals and humans have been enacted in the United States and Europe due to the concern for AF-induced toxicity. Feed for mature, non-lactating animals has been assigned an FDA action limit of 100 ppb total AFs in the U.S. and commodities destined for human consumption cannot exceed 20 ppb. Due to high consumption of milk by infants and children the FDA action level for AFM_1 in milk products, has been lowered to 0.5 ppb. Although regulations on AF content have been beneficial in U.S. and European markets, such regulations are lacking in most African countries. It is also important to note that strict European and U.S. regulations have influenced exportation of grains and oilseeds from Africa, resulting in farmers keeping the lower quality produce for their own consumption.

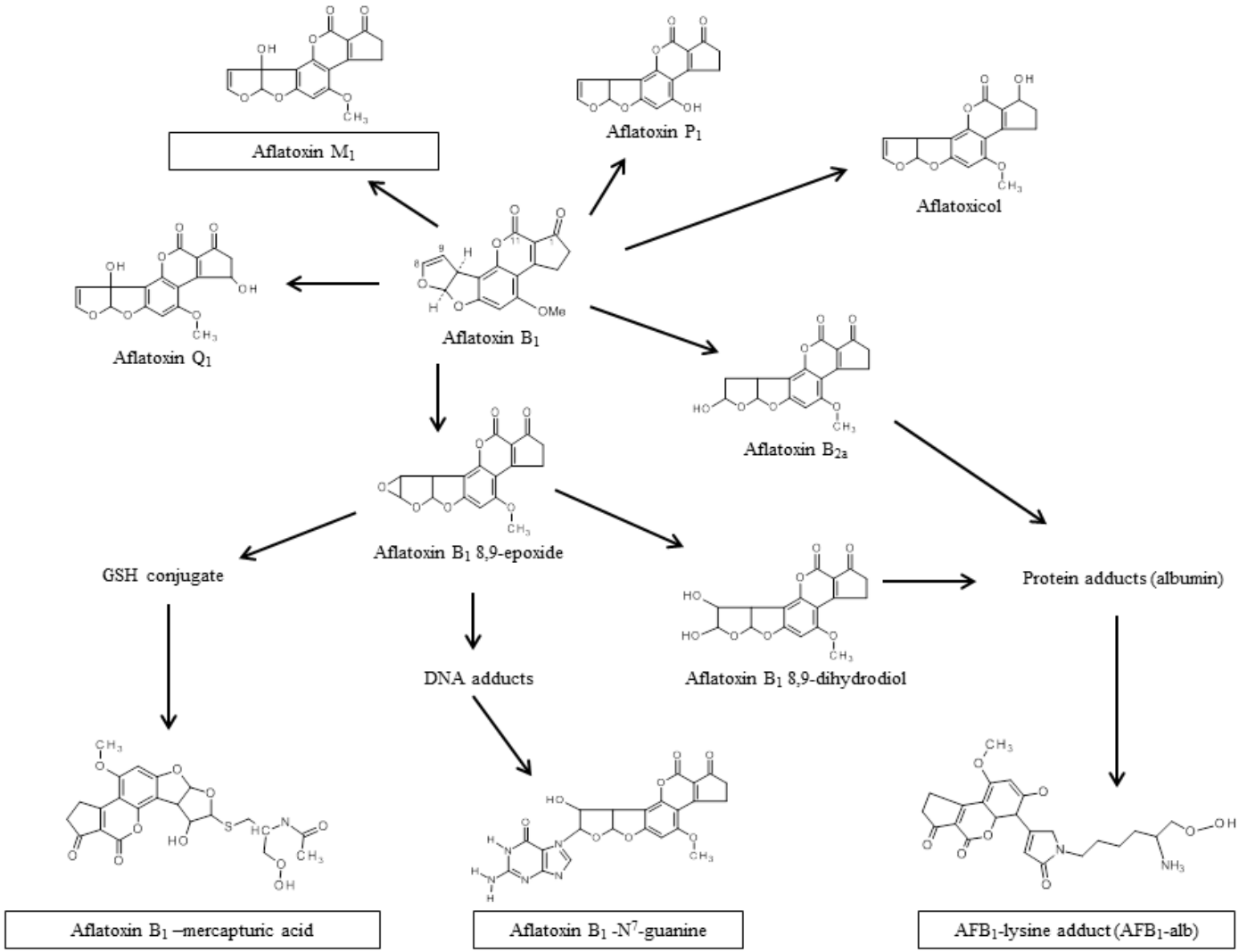


Figure 2. AFB_1 metabolism. Highlighted boxes indicate linear biomarkers of exposure. GSH = Glutathione. Adapted from Eaton et al. (1994).

1.2 Fumonisin

1.2.1 Fumonisin chemistry

Fumonisins (FBs) are a recently discovered family of mycotoxins, consisting of 15 structurally similar analogs produced by *Fusarium verticilliodies (moniliforme)* and *F. proliferatum* (Marin S et al. 2004). While *Fusarium* fungi, identified by John Sheldon in 1904, was known to cause adverse health effects in animals, the toxic agent was not elucidated until many years later. During an investigation of increased esophageal cancer incidence in South Africa, scientists elucidated the structure of FBs and implicated them as the source of toxicity (Gelderblom et al. 1988). It was later discovered that the source of the described FBs was *F. verticilliodies*. FBs contain a long carbon-chain backbone with two tricarboxylic acid groups esterified at C14 and C15. The tricarboxylic acid and amine groups on the carbon-chain make the compounds highly water soluble (Figure 3). The fumonisin B_1 (FB_1) isoform is the most studied and is frequently implicated in the manifestation of multiple animal and human diseases and thus will be the toxin of interest in this text. The stereochemistry of FBs is still unknown. However, it has been proposed that the acid side chains can act with the backbone, causing the molecule to fold on itself creating a spherical globular model. This model suggests that FB_1 could be a potential chelator of ions important for cellular function, such as potassium and calcium (Beier and Stanker 1997).

1.2.2 Source of fumonisin contamination

Ear rot and kernel disease in maize most frequently results from infection with *Fusarium* fungi (Marasas et al. 1984). *Fusarium* species are primarily associated with

infection of maize crops but can also affect wheat and other cereal grains (Marasas 1995). Two factors that contribute to the growth of *F. verticilliodies* and *F. proliferatum*, and the production of FBs, are water activity (a_w) and temperature (Doohan et al. 2003). Climate affects not only growth and germination, but production and dispersal of *Fusarium* species as well. *F. verticilliodies* and *F. proliferatum* are known to reproduce both sexually and asexually (Leslie 1996; Parry et al. 1995). Optimal temperatures for germination are between 25-37°C, and a minimum a_w of 0.88 is required (Marin S et al. 1996). Water activity defines the intensity of association between water and a non-aqueous substance and typically rises with temperature. It is calculated as a ratio of vapor pressure of water in a solid material (i.e. maize) to vapor pressure of pure water, thus an a_w of 0.88 means the vapor pressure of the maize is 88% that of pure water. A high a_w value indicates a high-risk for bacterial, yeast, and fungal growth. Optimal conditions for growth of *F. verticilliodies* and *F. proliferatum* on maize were reported at 30°C and an increasing a_w (>0.925) (Marin S et al. 1995). Although *Fusarium* is one fungi that is often attributed to global contamination, the two species known to produce FBs, *F. verticilliodies* and *F. proliferatum,* grow best in high temperatures (Keller et al. 1997; Kostecki et al. 1999; Marin S et al. 1999a; Miller 2001). FB production by these fungi is more highly dependent on a_w than temperature; the temperature range for optimal FB production can range between 15-30°C (Cahagnier et al. 1995; Marin S et al. 1999a). In general, *F. verticilliodies* FB production is greatest at 20-25°C and a_w 0.98, however *F. proliferatum* prefers lower temperatures around 15°C (Marin S et al. 2004).

Thus, reports of high prevalence of *Fusarium* infection and FB exposure in tropical and sub-tropical climates, where the relative humidity is highest, are not unexpected.

1.2.3 Fumonisin toxicity

FB_1 has a complex mode of action and biological effects have been difficult to extrapolate to other species, particularly humans, due to gender, strain, and species differences in effects (Muller et al. 2012). The complexity of FB toxicity can be fully appreciated when assessing variability in species sensitivity to the toxin. Horses and swine are the most sensitive species to FB exposure. Horses may develop equine leukoencephalomalacia (ELEM) following the ingestion of as little as 5-10 ppm. ELEM is characterized by necrosis of the brain white matter leading to signs such as ataxia, trembling, anorexia, and death (Kellerman et al. 1990; Marasas et al. 1988; Uhlinger 1997). However, toxicity manifests differently in swine with the development of porcine pulmonary edema following FB_1 ingestion, frequently resulting in death. The exact mechanism by which pulmonary edema develops in swine is unknown; however, there is evidence that FB_1 acts as a cardiotoxin through the increased production of TNF-α (He et al. 2001; Soriano et al. 2005). It is also suggested that the disruption of sphingolipid metabolism by FB_1 causes collapse of cell to cell junctions leading to degradation of the endothelial barrier and formation of "leaky" vessels, which results in hypertension (Ramasamy et al. 1995).

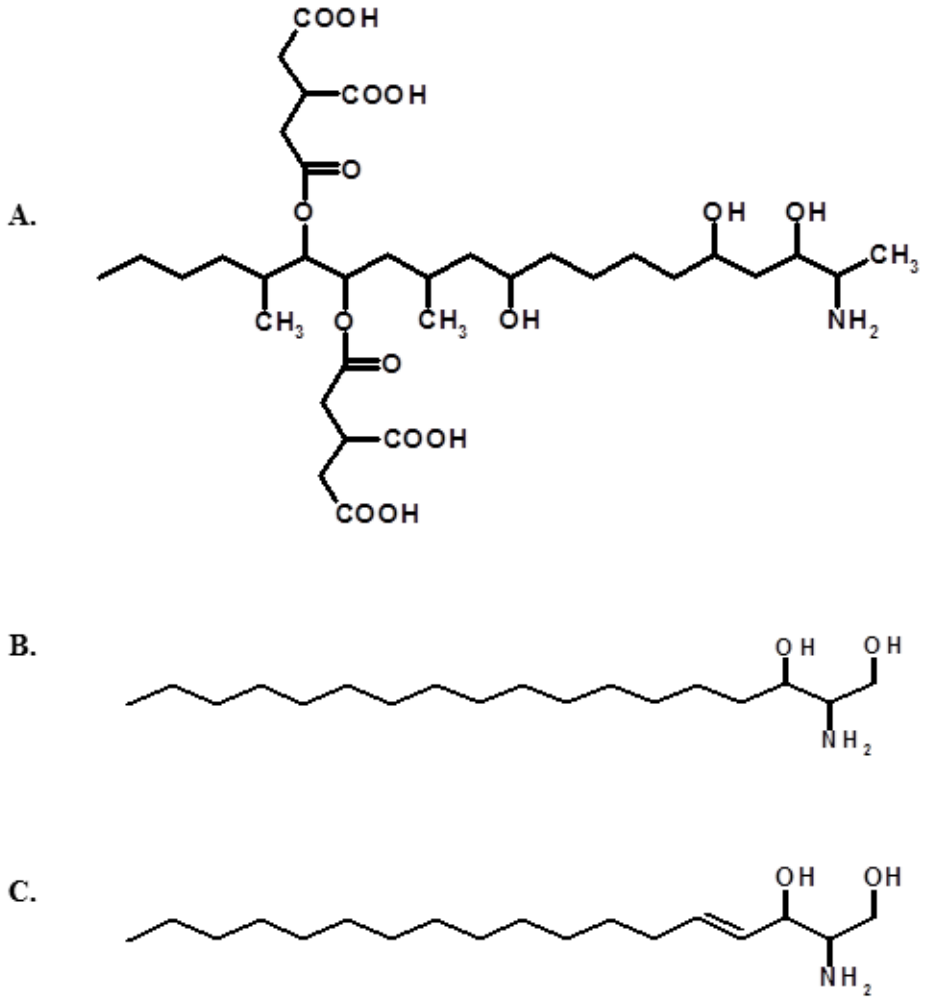


Figure 3. Fumonisin chemical structure. A) fumonisin B_1, B) sphinganine, C) sphingosine

During multiple rodent toxicity assays conducted by the U.S. Department of Health National Toxicology Program, the most common toxic endpoints identified following FB_1 treatment was renal carcinogenicity and nephrotoxicity (NTP 2001). Target organs for FB_1 toxicity are the kidney and liver in rodent models. Acute toxicity of FB_1 appears to be low but at a dose of 150 ppm, for 4 weeks in Sprague-Dawley rats, significant increases in triglycerides, cholesterol, and alkaline phosphatase (ALP) were observed indicating hepatotoxicity under this treatment regimen (Voss et al. 1993a). Cortical nephrosis was also observed in animals given lower doses of FB_1, however this study also indicated that males were more sensitive to FB_1-induced renal toxicity than female Sprague-Dawley rats. The same kidney lesions, tubular epithelial basophilia and cell degeneration in the proximal convoluted tubules, did not occur in females until a dose of 50 ppm, while males developed these histopathological effects after 15 ppm FB_1. Although Fischer-344 rats (F344) demonstrated similar sex-linked sensitivities to FB_1 nephrotoxicity as the Sprague-Dawley rats, hepatic apoptosis occurred more frequently in females of this breed (NTP 2001). In contrast, it was reported that FB_1 treatment in B6C3F/Nctr BR mice, at the same doses given to rats (up to 56 mg/kg), does not result in nephrotoxicity, but causes hepatocellular effects such as, necrosis, periportal hypertrophy, and centrilobular and bile duct hyperplasia (NTP 2001). Increased levels of alanine aminotransferase (ALT) and ALP in the serum also indicated hepatotoxic effects of FB_1 and, like rats; females were more sensitive to the hepatic toxicity than males. Interestingly, male BDIX mice, dosed with *F. moniliforme* culture, showed signs of hepatotoxicity but not nephrotoxicity. Gelderblom et al. (1991) discovered fibrosis,

degeneration, and proliferation of the hepatobiliary tract in male mice given FB treatment but found minimal changes in the kidney. Although, the toxins produced from the *F. moniliforme* cultures used in this study were not defined, this is one of the first studies to demonstrate the possible carcinogenicity of FB. Gender-differences in susceptibility are also prevalent among other species. Male pigs are more highly affected by FB_1 exposure than females. FB_1 treatment in weanling pigs decreased antibody levels following vaccination and expression of IL-10 in males only (Marin DE et al. 2006). Similarly, males were more sensitive to toxicity as evidenced by higher levels of free sphingolipids in the lungs (the target organ in swine) when compared to effects in females (Rotter et al. 1996). Sub-chronic exposure assessment in F344 rats confirmed the kidney as a target organ in this species and its gender-specific variability (Voss et al. 1995).

Evidence of renal carcinogenicity in F344 rats occurred in males following a two year treatment protocol (NTP 2001). Doses of 50 and 150 ppm FB_1 included in the diet resulted in renal tubular adenomas, carcinomas, and increases in preneoplastic lesions. Renal carcinomas were highly metastatic; metastasis to the lung occurred in 50% of rats at the highest dose of FB_1. Renal tubular carcinomas showed a malignant anaplastic phenotype that was highly invasive and mitotic (Hard et al. 2001). The incidence of hepatocellular neoplasms were higher in females than males in a study conducted by the same group utilizing B6C3F/Nctr mice (NTP 2001). FB_1-mediated toxicity studies lasting for two years in BDIX mice resulted in increased hepatocyte nodules and HCC (Gelderblom et al. 1991; Gelderblom et al. 1996b). Interestingly, the authors also

reported hepatotoxic and nephrotoxic non-neoplastic lesions at lower doses that did not induce carcinogenic effects. Based on these chronic studies a no observed effect level (NOEL) of 0.67 mg/kg b.w. per day was defined for tumor induction in rats and 0.22 mg/kg for renal toxicity (Howard et al. 2001; NTP 2001).

1.2.4 Mechanism of action

FB_1 is poorly absorbed by the GI tract, only 4% of an orally administered dose was recovered in the plasma and tissues of rats (Martinez-Larranaga et al. 1999). FB_1 is rapidly distributed to the liver and kidney and eliminated with a plasma half-life of only 3.15 hr Treatment of Sprague-Dawley rats for ten days showed accumulation of FB_1 in the kidney and liver, although the concentration in the kidney was 10-fold higher than the liver (Riley and Voss 2006). Importantly, the concentration of FB_1 in the kidney positively correlated with histopathological changes observed in the kidneys. Multiple studies designed to assess the metabolism and biotransformation of FB_1, *in vitro* and *in vivo*, have indicated that FB_1 is not biologically activated or deactivated following ingestion. Unlike AFB_1, CYP450 enzymes do not metabolize FB_1. However, a hydrolyzed form of FB_1 (HFB_1) is excreted in the feces (Shephard et al. 1995, 1994). The HFB_1 excreted product is not a result of direct biological metabolism; it results from microbial deesterification of the tricarboxylic acid groups in the gut and not well absorbed. Therefore, although HFB_1 is more toxic than FB_1 following intraperitoneal administration (Seiferlein et al. 2007), it is not thought to contribute to FBs toxicity outside of possible GI effects (Humpf et al. 1998). Absorbed FB_1 primarily remains in its non-metabolized form and is rapidly excreted in the feces (primary route) or urine.

Structurally, the 3,5-dihydroxy, 2-aminoalkane backbone of FB_1 is similar to endogenous sphingolipids, sphinganine and sphingosine; this likeness is believed to be the basis of their toxicity (Merrill et al. 1996) (Figure 4). The backbone similarity to natural substrates of sphinganine N-acetyltransferase (ceramide synthase) led to research involving the inhibition of this important enzyme by FB_1. Its suspected mode of action is described in Figure 4. Ceramide synthase is an integral enzyme in cellular sphingolipid metabolism by acetylating free sphinganine to produce dihydroceramide during *de novo* sphingolipid synthesis. This enzyme also catalyzes deactylation of sphingosine to ceramide (Mullen et al. 2012; Pewzner-Jung et al. 2006). There is evidence that sphingolipids are not solely utilized as structural units of biological membranes, but are involved in various intracellular and extracellular functions including differentiation, growth, proliferation, senescence, apoptosis, and necrosis (Adam et al. 2002; Okazaki et al. 1989; Ruvolo 2003; Venable et al. 1995; Wang G et al. 2005). FB_1 potency is influenced by the concentration of sphinganine, suggesting that FB_1 most likely interacts with sphinganine binding sites within ceramide synthase (Merrill et al. 2001). Inhibition of ceramide synthase by FB_1 results in an accumulation of free sphinganine in serum and tissues and decreases in ceramide and complex sphingolipids (Merrill et al. 2001; Riley et al. 2001; Wang E et al. 1991). Consequently, FB_1 exposure has been correlated with increases in free sphinganine levels in the blood and urine resulting in an unbalanced sphingolipid ratio (Gelderblom et al. 1996a; Riley et al. 1993, 1994; Wang E et al. 1992). Importantly, Riley et al. (1994) observed a strong dose-response relationship between FB_1 treatment in rats and sphingoid base levels in the kidney prior to the onset

of renal toxicity, indicating a possible mechanistic basis for disrupted sphingolipid metabolism and FB_1-induced toxicity. Sphinganine and sphingosine are known to cause cytotoxic, apoptotic, and inhibitory effects on cells when applied exogenously, therefore accumulation of these sphingoid bases following FB_1 exposure could be responsible for apoptosis observed during toxicity studies (Muller et al. 2012). Although there is evidence to support the hypothesis that FB_1-mediated toxicity is a result of inhibition of ceramide synthase activity and accumulation of sphingoid bases and decreases of more complex sphingolipids, more recent work suggests that additional targets also contribute to the cellular response from FB_1 exposure (He et al. 2001; Riley and Voss 2006; Seefelder et al. 2003).

The potential carcinogenicity of FB_1 stimulated research on the genotoxicity of FB and there is evidence that although FB_1 is not a genotoxic compound it does acts as an indirect carcinogen. Mutagenicity studies with *Escherichia coli* and *Salmonella typhimurium* have repeatedly shown negative results (Aranda et al. 2000; Gelderblom and Snyman 1991; Knasmuller et al. 1997). However, DNA damage, including strand breaks, have been observed by the comet assay in numerous mammalian cell lines and *in vivo* in both liver and kidney of rats (Atroshi et al. 1999; Domijan A et al. 2006, 2007, 2008; Ehrlich et al. 2002; Galvano et al. 2002a, 2002b). The lack of FB_1 reactivity with DNA has led to conclusions that the DNA damage observed in these studies are mediated through oxidative stress resulting from FB_1 exposure. In rats, FB ingestion causes accumulation of fatty acids in the liver, which are subject to lipid peroxidation, free radical formation and damage leading to cell transformation (Gelderblom et al.

1996a). Reactive oxygen species (ROS) act as highly reactive electrophiles and can cause damage to proteins, lipids, and DNA. FB_1-mediated ROS production has been implicated as the causative agent in DNA damage and carcinogenic potential of FB_1 in biological systems (Abel and Gelderblom 1998; Domijan A et al. 2007, 2008; Kouadio et al. 2007). FB_1 treated rats had DNA lesions in their kidney in addition to signs of oxidative stress (i.e. increases in protein carbonyls and malondialdehyde). *In vitro* studies have supported these findings; porcine and monkey kidney cells showed increased levels of malondialdehyde following FB_1 treatment (Abado-Becognee et al. 1998; Klaric et al. 2007; Meca et al. 2010). Interestingly, FB_1 has also been associated with depleted levels of an important cellular ROS scavenger, GSH, further supporting an increase in ROS species following FB_1 induced toxicity (Atroshi et al. 1999; Bondy et al. 1995; Kang and Alexander 1996). However, there is still no conclusive evidence whether lipid peroxidation and oxidative stress is an indirect or direct consequence of FB_1 toxicity (Abel and Gelderblom 1998; Muller et al. 2012).

1.2.5 Human exposure in Africa

Human exposure in Africa occurs through consumption of contaminated maize meal. Consumption of FB_1, like AFB_1, is highest in rural communities of South America, Africa, and Southeast Asia, due to a favorable climate for fungal growth, limited.

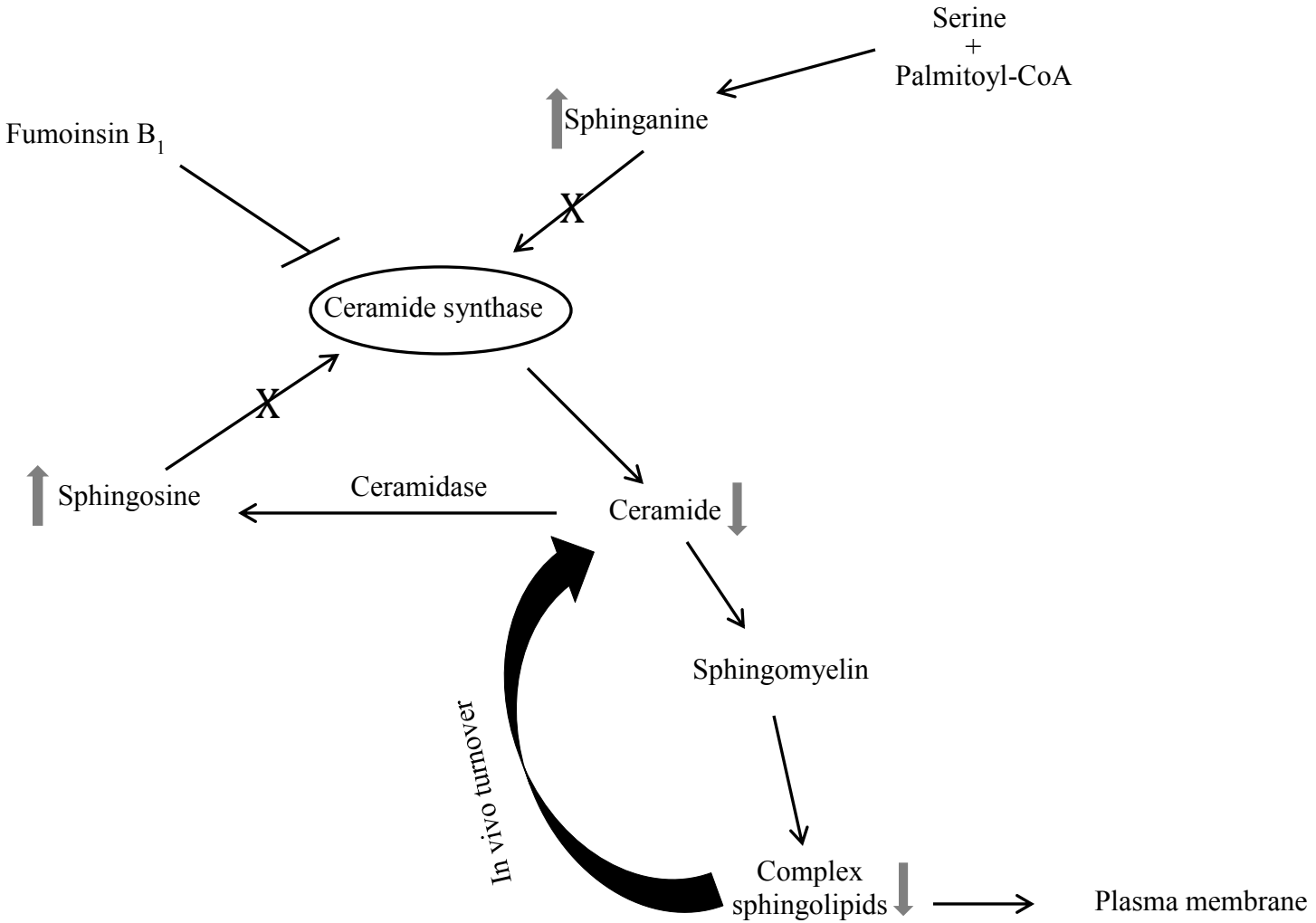


Figure 4. A model of de novo sphingolipid biosynthesis and inhibition by fumonisin B_1. The gray arrows indicate known effects of pools of metabolites resulting from fumonisin B_1 inhibition of ceramide synthase. Reductions in complex sphingolipids can disrupt the integrity of the plasma membrane of cell. Adapted from Riley and Voss 2006.

variety in the diet, and unsuitable cultivation and storage practices. It has been estimated that dietary intake of FB_1 could be as high as 354.9 μg/kg bw per day, in areas of Africa consuming large quantities of maize based foods (Muller et al. 2012). In humans, the most widely recognized effect associated with the consumption of FB_1-contaminated corn is the development of esophageal squamous cell carcinoma (Alizadeh et al. 2012; Chu and Li 1994; Gong HZ et al. 2009; Rheeder et al. 1992; Williams et al. 2010). Although FB_1 is associated with carcinomas in many species, it does not induce genotoxicity in cellular models, resulting in a 2B classification (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC 2002). Lack of an appropriate biomarker of FB_1 exposure has limited the findings and efforts of epidemiological based studies correlating FB_1 with human based health effects. Thus, most reports are limited to generalizations of populations with a higher incidence of a specific disease who also consume large amounts of maize, or maize from the same region containing FB_1 contamination. A recent report in China showed no correlation between FB_1 levels in the toe nails of participants and HCC incidence, following adjustments for HBsAg positive, sex, age, and alcohol consumption status (Persson et al. 2012). However, the biomarker utilized in this study was not validated for linearity with FB_1 exposure in humans or animal models. While epidemiological evidence for carcinogenic potential of FB_1 in human populations is lacking, animal models indicate that FB_1 could be a contributing variable and additional risk factor for development of cancer, particularly in populations at high-risk for cancer development due to other environmental factors. Of concern is FB exposure in populations in Ghana, where HBV

infection and AF exposure is endemic. Up to 72.1% of participant urine samples were positive for FB_1 in a rural community of Ghana (Robinson et al. 2012), demonstrating a high incidence of exposure to this mycotoxin in communities at high-risk for HCC development.

Recently, FBs have also been proposed as a risk factor for neural tube defects (NTDs) in areas where populations consume large quantities of corn and corn based foods (Gelineau-van Waes et al. 2009; Missmer et al. 2006; Suarez et al. 2012). The incidence of NTDs is heightened in areas where corn is a dietary staple and FB contamination is common, such as in Guatemala, Northern China, and the Transkei region of South Africa. These areas have reported an incidence of NTDs that is 6-10 times higher than the global average (Hendricks K 1999; Marasas et al. 2004; Moore et al. 1997; Ncayiyana 1986; Xiao et al. 1990). In the U. S., corn crops along the Texas-Mexico border registered unusually high levels of FB contamination during 1990-1991, which correlated with substantially higher NTDs (2.7/1000 live births) compared to the same area in 1986-1989 (1.5/1000 live births) (Hendricks K 1999; Missmer et al. 2006). While a mechanism of action has not yet been delineated, FB_1 appears to inhibit uptake and metabolism of folic acid, a vitamin essential for normal fetal NT development.

Like AF, FBs have been implicated in a myriad of biological endpoints that are not fully understood. Potential health effects of FBs in humans are still being assessed. For example, Kimanya et al. (2010) recently reported that children from Tanzania consuming foods contaminated with greater than 2 μg/kg bw FB were significantly shorter and lighter than children with lower FB_1 exposure. This implies that FB_1

exposure may have a possible association with growth faltering in Africa. Development of an appropriate biomarker and further research in the mechanism of action for FB_1 would improve the quality of epidemiological based studies.

1.2.6 Biomarkers of exposure

Development of a stable, linear, dose-dependent biomarker of FB exposure has proven difficult due to its short half-life in the serum and urine as well as a lack of detectable metabolites. FB_1 is known to affect sphingoid base concentrations as a result of ceramide synthase inhibition and was thus the first biomarker to be investigated. An increase in the sphingolipid ratio (sphinganine/sphingosine) is linear with increased consumption of FB_1 in various animal models and has often been utilized to assess exposure in animal species (Cai et al. 2007; Shephard et al. 1996). However, this biomarker of exposure is not linear within humans (Qiu and Liu 2001; Solfrizzo et al. 2004; van der Westhuizen et al. 1999), which is likely due to genetic variations in lipid production. Parent FB_1, was proposed as a biomarker for determining FB exposure in humans (Shephard et al. 1995, 2007). Cai et al. (2007) concluded that excretion of parent FB_1 in the urine following repeated dosing was strongly correlated with urinary sphingolipid metabolites and serum sphinganine/sphingosine ratio and should be further assessed for linearity with human FB_1 intake. The first human study to report a method for urinary FB_1 analysis that showed a positive correlation with FB exposure and FB_1 excretion was conducted in a Mexican population consuming large quantities of maize-based tortillas (Gong YY et al. 2008). Following this study, researchers from the PROMEC Unit in South Africa confirmed this correlation, with a correlation coefficient

of 0.4254 and p-value<0.01 (van der Westhuizen et al. 2011). Interestingly, the percentage of FB_1 consumed that was excreted in the urine ranged from 0.054 to 0.104% per day, significantly lower than that proposed from animal studies (up to 2%). Riley et al. (2012) investigated the kinetics of urinary FB_1 excretions in humans consuming maize based diets from Guatemala. Average excretion of FB_1 in this study accounted for 0.12 to 0.9% of the FB_1 intake, however a correlation between intake and excretion was not reported. Variability in FB_1 excretion was high between individuals and was cleared within 72 hr of exposure. Therefore, use of urinary FB_1 levels in epidemiological based studies should consist of large population sizes and multiple samplings from the same participant to account for inter-individual variations in excretion.

1.2.7 Regulation

The occurrence of FBs in corn is ubiquitous, causing concern for populations where corn is a staple commodity used in a variety of foods. Currently there is insufficient information to adequately assess risk in populations that consume high amounts of corn. However, the U.S. FDA has established recommended maximum levels for FBs in animal and human foods, which are much higher than those established for AFs. A 60 ppm action level has been recommended for feeds for ruminant slaughter animals. Corn products for human consumption have been limited to 4 ppm for total FBs. JECFA has set a provisional maximum tolerable daily intake (PMTDI) for FB_1, FB_2, and FB_3 combinations of 2 ppm per day.

1.3 AFB_1/FB_1 co-exposure

Discovery of similar biological endpoints, such as liver toxicity, immunotoxicity, and growth faltering for AFB_1 and FB_1 exposure has led to public health concerns in communities that are dually exposed to FBs and AFs. A high-risk for AF and FB co-contamination in maize-based products is common in climates favorable for growth of both *aspergillus* and *fusarium*. Surveys of maize flour from markets and households in Ghana have reported mixtures of both AFs and FBs (Kimanya et al. 2008; Kpodo et al. 2000; Kumi et al. 2011). Additionally, biomarkers for both toxins were found in high frequency in an adult population residing in rural Ghana (Robinson et al. 2012; Wang P et al. 2008). Dual exposure risk has led to a recent assessment of possible antagonistc, additive, or synergistic effects of AFB_1/FB_1 mixtures on toxicity. Acute toxicity evaluation of combinations of AFB_1 and FB_1 demonstrated an additive effect in mortality in F344 rats (McKean et al. 2006). During this study various fractions (1.0, 3/4, 1/2, 3/8, 1/4, 1/8) of the AFB_1 LD_{50} value (2.71 mg/kg) for these rats was mixed with 25 mg/kg FB_1 and dosed by gavage. Acute toxic effects such as depression and diarrhea occurred within a few hr of the treatment in the four highest doses, while no effect was observed in the two lowest doses. Importantly, mortality was 100% in the two highest doses (2.71 mg/kg AFB_1 + 25 mg/kg FB_1 and 2.03 mg/kg AFB_1 + 25 mg/kg FB_1) 72 hr after treatment, while treatment of 2.15 mg/kg AFB_1 alone only had a 20% mortality rate during a one week study period (McKean et al. 2006). An interaction index (K) of 1.98 was calculated for combined toxicity indicating an additive interaction of AFB_1 and FB_1. Similarly, the same research group found a K value of 1.98 in

AFB_1/FB_1 combinative toxicity analysis in mosquito fish as well. Results from AFB_1/FB_1 toxicity in *Hydra vulgaris*, showed the combination to be more toxic than either mycotoxin alone (Brown KA et al. 2012). Following exposure to the mixture of AFB_1 and FB_1, all hydra had disintegrated. Although the single AFB_1 or FB_1 treatments caused morphological alterations the hydra were still viable. Male Wistar rats fed AFB_1/FB_1 contaminated diets had higher numbers of tubular apoptotic cells in the kidney compared to those treated with either FB_1 or AFB_1 alone (Theumer et al. 2010; Theumer et al. 2008). Additive effects were also observed in the liver; only the mixture group had signs of cellular mitosis and apoptosis after treatment. It is interesting to note that the increase in mitotic and apoptotic conditions in the liver of AFB_1/FB_1 supports evidence found by Gelderblom et al. (2002) even though the treatment protocols were vastly different. Theumer et al. (2008) dosed animals with AFB_1 and FB_1 at the same time, while Gelderblom and associates designed a sequential dosing regimen due to the cancer initiation and promotion potentials of AFB_1 and FB_1, respectively. AFB_1 and FB_1 sequentially treated F344 rats had higher weight gain and relative liver weights than those treated with FB_1 alone, however the AFB_1 group had similar weights compared to the mixture group (Gelderblom et al. 2002). The AFB_1/FB_1 treatment increased the size and number of the placental form of glutathione-S-transferase positive ($GSTP^+$) foci in the liver compared to either individual mycotoxin following promotion with 2-acetylaminofluorene and partial hepatectomy (2-AAF/PH). Co-treatment also induced larger amounts of cellular apoptosis and mitoses and cirrhotic livers with numerous dysplastic nodules. The authors concluded that not only can FB_1 act as a promoter of

AFB_1-induced hepatocytes, but AFB_1 enhanced the potency of FB_1, presumably by increasing the susceptibility of the liver to fumoinsin's toxicity (Gelderblom et al. 2002).

One study conducted in weaned swine showed no additive effects of FB_1/AFB_1 co-exposure except for a decrease in feed consumption and feed conversion (Dilkin et al. 2003). However, the dose of AFB_1 given in this study was very low (50 ug/kg) considering the LD_{50} value in weanling swine is 620 ug/kg (Cullen and Newberne 1994). Additionally, the species differences in effects of FB_1 exposure in pigs would indicate that AF and FB may not act additively or synergistically in this species due to a difference in target organs (i.e. lung and liver, respectively). An alternate study in swine utilizing 2.5mg/kg AFB_1 and 100 mg/kg FB_1 did indicate additive and possible synergistic effects of the combination (Harvey et al. 1995). Additive effects included increases in body weight and hematologic measurements, while cholinesterase and ALP levels were synergistically elevated. The authors concluded that the combination diet of AFB_1 and FB_1 was at least additive for toxicity, and particularly for incidence of liver disease.

1.4 Methods to reduce exposure in human populations

AF toxicity, exposure, and intervention strategies have been investigated for over 50 years. However, there is still approximately 4.5-5 billion of the world's population exposed to AFs (Strosnider et al. 2006; Williams et al. 2004). Intervention strategies to reduce this exposure have been an important topic in mycotoxicology for 20 to 30 years. Although there have been numerous proposed mechanisms, humans and animals are frequently exposed to high levels of AFs, particularly in rural areas of developing

countries. Strategies to reduce mycotoxin exposure in these high risk areas can be categorized into one of three approaches: 1) Pre-harvest (agricultural), 2) Post-harvest (storage), and 3) Chemoprotective. Pre-harvest interventions include technologies that are utilized in the field to reduce or eliminate *Aspergillus* contamination and growth. Development of transgenic strains of maize and peanut crops resistant to *Aspergillus* has been developed and may provide both health and economic benefits in communities at risk (Guo et al. 2008; Holbrook et al. 2009; Menkir et al. 2006). Insect damage in the field is a main factor that can predispose maize crops to fungal contamination via promoting colonization through kernel injury and acting as vectors for fungal spores. Transgenic maize that is insect resistant has also been applied in areas of high AF incidence, however there were mixed results in its efficacy (Wu 2006). Similar, pre-harvest control includes atoxigenic strains of Aspergilli that reduce *Aspergillus* colonization on crops through competition. These competitive fungi have shown a decrease of 70 to 99% following co-inoculation of African maize (Atehnkeng et al. 2008). Good agricultural practices for post-harvest storage in the industrialized world prevent accumulation of mycotoxins. However, in developing countries storage practices tend to be rudimentary and can promote the growth of fungi and production of mycotoxins. Education of farmers in different agricultural processes can be effective in reducing levels of mycotoxins. In particular, early harvesting, proper drying, proper storage (proper ventilation), and insect management have shown improvement in contamination (Wagacha and Muthomi 2008; Wu 2008). Hand sorting of AF contaminated kernels can also be effective in reducing exposure in high-risk areas

(Kabak et al. 2006). Although these types of interventions improve food quality, implementation is often short lived in rural areas after research teams exit the community. Strategies to utilize crops that have been contaminated in the field or during storage are often of concern for mycotoxicologists working in Africa and Southeast Asia. Communities that are at highest risk for mycotoxin exposure are often forced to consume lower quality crops due to food insecurity. Disposing of any food is both culturally unacceptable and economically unfeasible.

Chemoprotective interventions would allow for either human or animal consumption of foods that would otherwise be considered unsuitable for consumption by decreasing the toxic effects of ingested mycotoxins. Chemoprotective interventions include chemical compounds that alter the metabolic activation of AFB_1. Three of the most studied strategies; green tea polyphenols (GTP), chlorophyllin, and a form of dithiolethiones named oltipraz are reviewed by Groopman et al. (2008). GTPs have been implicated as inhibitors of carcinogenesis from various chemical compounds in animal models as reviewed by Yang et al. (2006). Inclusion of GTP in the water of AFB_1 treated rats decreased the number of preneoplastic lesions in the liver by 60-70% and inhibited AFB_1-DNA binding by 20-30% (Qin et al. 1997). Similarly, GTP reduced oxidative DNA damage during a Phase IIa clinical trial in a population at high-risk for AF exposure in China (Luo et al. 2006). Chlorophyllin has been effective in inhibiting HCC in AFB_1-treated trout (Breinholt et al. 1995a) and appears to both sequester AF and induce metabolic enzymes (Breinholt et al. 1995b; Fahey et al. 2005). Reductions in AFB_1 biomarkers have confirmed efficacy of chlorophyllin in rats and a human

intervention trial (Egner et al. 2001; Simonich et al. 2007). Oltipraz acts to modify drug-metabolizing enzymes through the Keap1-Nrf2 complex (Groopman et al. 2008). Nrf2 is a transcription factor that acts through the antioxidant response element. Keap1 acts to sequester Nrf2 in the cytoplasm; dissociation of the Keap1-Nrf2 complex results in translocation of Nrf2 to the nucleus and induction of transcription of various phase II detoxifying enzymes (Dinkova-Kostova et al. 2005; Kwak et al. 2004). One month intervention with Oltipraz in a human population reduced the urinary AFM_1 biomarker by 51% and increased AF conjugation through GSTs (Kensler et al. 1998; Wang JS et al. 1999). However, all these methods take numerous weeks, months, or even years to reduce AF exposure and would not be useful for acute outbreaks such as that seen in Kenya in 2004. Research in our laboratory has focused on the safety and efficacy of inclusion of a natural clay product that acts to bind AF and possibly FB, reducing their bioavailability in the GI tract. Thus, it is proposed that this product would be useful during instances of high AF exposure in populations presented with epidemics of jaundice, lethargy, or acute death from hemorrhaging of the liver.

1.4.1 Enterosorption with dioctahedral smectite clays

Smectite is one of the major mineral groups of phylosillicate clay fractions from soils (Reid-Soukup and Ulery 2002). They occur naturally in environments with high silicon and basic cation activities and are widely distributed around the world. Smectites have unique layered structures often described as a deck of cards randomly thrown on a table. Generally, they consist of two tetrahedral sheets (silicon oxide) and one octahedral sheet (aluminum oxide) to comprise a 2:1 lattice layer with interlayer regions between

each 2:1 lattice (Figure 5). The tetrahedral sheet is comprised of a Si^{4+} ion coordinated by four O^{2-} ions and the octahedral sheet contains Al^{3+} ions coordinated by six O^{2-} or OH^{-} ions. The oxygen units of these tetrahedral and octahedral structures can be shared creating a variety of structures (Schulze 1989). Their structure makes them highly adsorbent materials that are often utilized as cleaning or purifying agents. The groups of smectites include montmorillonite, beidellite, nontronite, hectorite, saponite, and sauconite. Montmorillonites are dioctahedrally coordinated; meaning that they are composed of one octahedral unit and two tetrahedral units on either side of the octahedral unit creating a sheet that is around 10 Å thick (Segad et al. 2010). One aluminum atom shares oxygen with the two silica sheets. Importantly, montmorillonites also naturally undergo isomorphic substitutions in the octahedral layer often exchanging Al^{3+} ions with Mg^{2+} and/or Fe^{2+}, creating a negative change on the platelets (Segad et al. 2010). Water and exchangeable cations, predominantly Ca^{2+} and/or Na^{+}, occupy the interlayer space between the platelets to equalize the negative charge. The physical and behavioral properties can change drastically depending on the major cation present in the interlayer space. Na^{+} montmorillonites generally have a larger swelling capacity than Ca^{2+} saturated clays. This is caused by stronger attractions of the platelet layers to the interlayer Ca^{2+} ions than the Na^{+} ions, resulting in less distance between the repeating platelets and reduced expansion of the interlayer space (Sato et al. 1992). Smectites have constant cation exchange capacities (charge on the surface of the clay platelet) ranging from 47 to 162 $cmol_c/kg$ (Borchardt 1989) due to the isomorphic substitutions present in the octahedral and tetrahedral sheets (Reid-Soukup and Ulery 2002). The particle size of

smectites is <0.2μm, comprising most of the fine clay fraction of soils, resulting in a large external surface area. Small particle size and interlayer expansion (swelling) promotes ion exchange and an increased surface area ranging from 600 to 800 m^2/g (Reid-Soukup and Ulery 2002). An interesting way to visualize the massive surface area of these types of clays was provided by Mitchell JK (1993). They calculated that 10 g of a dispersed Na-montmorillonite would cover an entire football field. All of these properties make montmorillonites good materials for absorbing positively charged materials, as seen by their ability to act as macronutrient reservoirs in soils; sequestering K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+} (Reid-Soukup and Ulery 2002). The sorbent properties of montmorillonites and the observation that populations at high-risk for exposure commonly engaged in geophagy (the consumption of soils) led to an investigation for the potential to bind mycotoxins with various natural clay materials. Pioneering work by Phillips et al. in the 1980s reported efficacy of a Ca-montmorillonite, NovaSil (NS) to decrease the negative health effects from AF exposure in multiple animal species. Further, molecular sorption analysis has proven that NS has a high binding capacity for AFB_1 *in vitro*.

1.4.2 Interaction between mycotoxins (AFB_1 and FB_1) and smectite

Isothermal analysis is a common method to investigate the sorption characteristics of a ligand on the surface of homogenous materials. Irving Langmuir developed a mathematical equation in 1916 that could describe the adsorption of gas molecules onto a solid surface, thereby significantly advancing the research of material sciences and adsorption (Langmuir 1916). The concepts of this modeling and

determination of binding capacity are described in detail by Kinniburgh (1986) and modified for mycotoxins by Grant (1998). Briefly, an isotherm is a plot of the concentration of a ligand left in solution versus the concentration bound to the surface of a solid. The shapes of the plots have been given classifications that describe the types of binding occurring (Giles et al. 1974a; Giles et al. 1960; Giles et al. 1974b). The plots can then be utilized in the Langmuir equation to determine the capacity (Q_{max}), affinity (K_d), and thermodynamics of adsorption.

AF binding analysis initially involved the use of ^{14}C or ^{3}H labeled AFB_1 to determine the binding capacity of NS clay at various pH values and temperatures. The AFB_1/NS complex was found to be stable at a variety of pH values and temperatures (Phillips et al. 1988). Following washing with various solvents, less than 10% of AFB_1 was extracted from the AFB_1/NS complex, suggesting a chemisorption reaction between AFB_1 and the surfaces of NS clay. Further research describing the mechanism of sorption indicated a high binding affinity and capacity of AFs on the surfaces of NS clay (Phillips et al. 1995). The index of chemisorption (Cα) was calculated from the initial concentration of AF, the amount bound, and amount desorbed and found to be relatively high (0.93). Similarly, calculation of enthalpy of the reaction was near or above -40 kJ/mol (Grant and Phillips 1998). In general, adsorption of materials onto solid surfaces can be classified as physisorption or chemisorption depending on the enthalpy of adsorption value (ΔH). Enthalpy values of less than -20 kJ/mol suggest a physisorption binding mechanism and chemisorption can be described as those reactions with a ΔH greater than -20 kJ/mol (Guo et al. 1994). Recent studies have reported that AFB_1

binding to smectite clays similar to NS involves direct ion-dipole interactions and electron sharing which explains the large heat of sorption described previously for NS (Deng et al. 2010). The maximum amount of AFB_1 able to bind with the surfaces of NS based on the Langmuir model has been calculated as 0.461 mol/kg (Q_{max}). Grant and Phillips (1998) demonstrated an actual binding capacity (Q_{max}) of 0.336 mol/kg. This indicates that a majority of the space available for binding is occupied by AFB_1, leading the authors to conclude that NS not only has a high binding capacity but also a high affinity for AFB_1. Importantly, the surface area of NS clay available for binding was determined to be 848 ± 11 m^2/g. AFB_1 binding with NS clay occurs primarily within the interlayer space of the clay platelets. Collapsing of the interlayer through heating the clay at 125° C for 12 hr resulted in a significant decrease in sorption of AFB_1 (Grant and Phillips 1998). Further work showed a similar AF binding capacity and affinity for a uniform particle size NS clay, or UPSN (Marroquín-Cardona et al. 2011).

1.4.3 Uniform Particle Size NovaSil (UPSN)

UPSN was developed by Texas Enterosorbents (Bastrop, TX, USA) as a refined NS clay for human use. The purification of parent NS was designed to selectively minimize the amount of particles >100μm in size (Marroquín-Cardona et al. 2011). This process was designed to reduce batch to batch variations in the crude NS clay

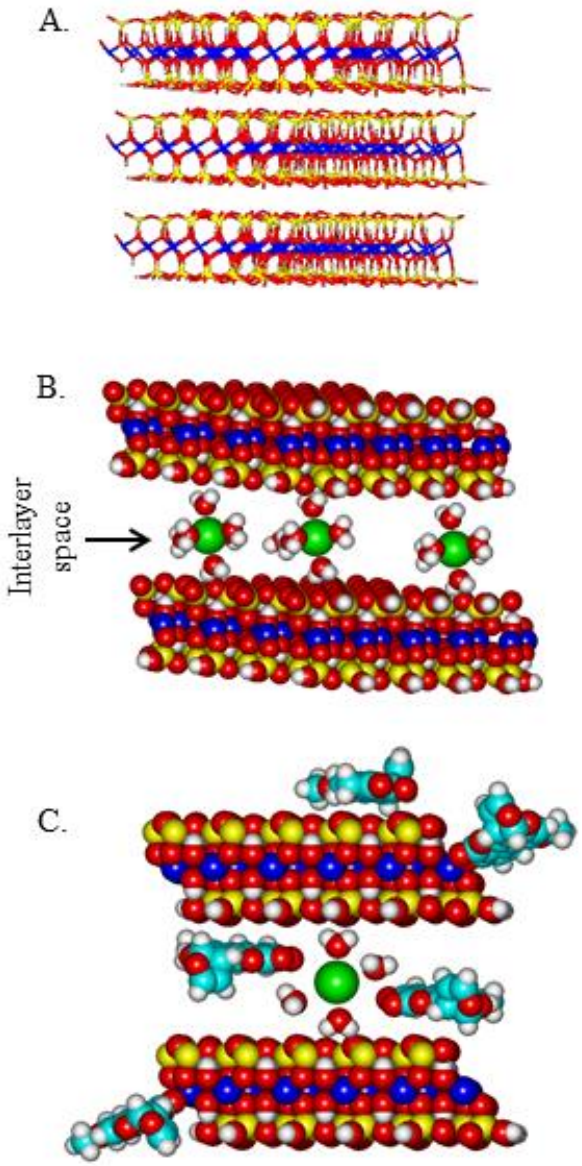


Figure 5. Montmorillonite structure: $Al_2Si_4O_{10}(OH)_2$. Schematic representation of 2:1 layer-lattice montmorillonite clay showing hydrated calcium as the predominant interlayer cation. Common substitutions: Mg^{2+} for Al^{3+}; Al^{3+} for Si^{4+}; and Fe^{3+} for Al^{3+}. Key: Si^{4+} (yellow), Al^{3+} (blue), O (red), H (white), C (light blue), and Ca^{2+} (green). A) Three-dimensional structure, B) Spatial structure demonstrating propping of the interlayer, C) AF bound structure.

making it more suitable for human consumption. In the dry state, UPSN contains a higher fraction of its particles in the 45-100 μm range (67%), while NS has a majority of particles smaller than 45 μm (50%) (Marroquín-Cardona et al. 2011). There were major differences in the distribution of particle sized between UPSN and NS, but Q_{max} and K_d parameters following isothermal analysis were similar for the two materials. Due to the similarities observed *in vitro*, it was speculated that UPSN would have comparable efficacy and safety *in vivo*. The majority of work presented in this dissertation will focus on human intervention strategies with UPSN.

1.4.4 Efficacy in animal and human models

The first studies showing efficacy of NS clay in reducing animal toxicity were conducted by a team at Texas A&M University and involved a variety of domestic farm animals. There is an extensive amount of literature describing the efficacy of montmorillonite clays to ameliorate the toxicity of various mycotoxins. A summary of these reports is shown in Table 1. For example, young broiler chicks administered 5 ppm AFB_1 and 0.5% (w/w) NS clay were protected from the growth inhibitory effects of AF observed in controls (Pimpukdee et al. 2004). In growing barrows, NS prevented toxicity of AF over a 4-week period, as determined by recovery of weight gain, serum alkaline phosphatase (ALP), and ɣ-glutamyl transferase (GGT) values as compared with control group levels (Harvey et al. 1994, 1989c). In another study, AF and AF+NS-treated lambs gained 8 and 92% of the weight that control animals gained over the course of the trial, indicating a protective index of 75% from NS treatment (Harvey et al. 1991a). Similarly, vitamin A levels in chicks were diminished following AFB_1 exposure, however 0.25 and

0.5% clay inclusion in the diet prevented AFB_1 effects on vitamin A levels in the liver (Pimpukdee et al. 2004). Leghorn chicks were also protected from the effects of 7.5 mg/kg AFB_1 with 0.5% clay inclusion (Phillips et al. 1988). Inclusion of a montmorillonite clay decreased the growth inhibitory effects of AFB_1 and gross pathological changes observed in the liver, such as a friable and pale appearance. A similar study conducted in Mexico confirmed these results. During this study chicks fed AF contaminated diets weighed 46% less than those receiving both AF and montmorillonite clay in their diet (Marquez Marquez and Tejada de Hernandez 1995). A montmorillonite clay (trademarked as Milbond-TX) was effective at a 1% dose in preventing reduced performance, changes in organ weights, serum chemistry changes, and gross pathology observed in broiler chicks fed 4 mg/kg AFB_1 for 21 days (Ledoux et al. 1999). Kubena et al. (1998) reported a decrease in the toxic effects of 5 mg/kg AFB_1 on weight gain in chicks by 43% with only 0.375% clay inclusion. Effects of AFB_1 on serum concentrations of cholesterol, albumin, triglycerides, calcium, glucose, and total protein were significantly reduced with 0.5% clay (Abo-Norag et al. 1995; Kubena et al. 1990a, 1993a, 1993b). A study conducted with various clay binders investigated the efficacy of clay to rescue broiler chicks from the immunomodulation observed following AF exposure for 21 days (Kececi et al. 1998). The increases in white blood cell count, and percentages of lymphocytes and monocytes caused by AF exposure were not completely recovered to control levels with clay, but were significantly lower than those treated with AF alone.

Turkey poults treated with 0.75 mg/kg AFB_1 and 0.5% montmorillonite clay demonstrated an average reduction of 52% in excreted AFM_1 when compared to the AFB_1 control animals (Edrington et al. 1996). Acute toxicity of 1 mg/kg AFB_1 was prevented in growing turkeys with treatment of clay at 0.5% (Kubena et al. 1991). The positive control group (1 mg/kg AFB_1 alone) had a mortality rate of 88%, while no deaths occurred in those turkeys receiving AFB_1 plus clay. One research group demonstrated acute toxicity of AFB_1 in mink; inducing 100% mortality at 102 µg/kg AFB_1 over 53 days of exposure (Bonna et al. 1991). Inclusion of 0.5% montmorillonite clay was effective in preventing mortality and toxic symptoms of aflatoxicosis. Weanling pigs have often been utilized as models for young children to assess possible health effects in humans. Studies have indicated that treatment with montmorillonite clay (NS) can reduce hepatic lesions, peripheral lobular lipidosis, peroportal and interlobular fibrosis, and bile duct hyperplasia induced by AFB_1 exposure (Harvey et al. 1989c). Impaired immune response following exposure to AFB_1 including, reduced mitogen-induced lymphoblastogenesis and peritoneal macrophage activity and function, was prevented in barrows receiving 0.5% clay along with AFB_1 (Harvey et al. 1994). Similar to the chicken studies described above, treatment with bentonite in AFB_1 exposed barrows, was effective in inhibiting growth stunting, decreased serum minerals, and decreased serum enzyme levels indicative of liver damage (AST, ALP, and gamma glutamyltransferase GGT) (Lindemann et al. 1993; Schell et al. 1993a, 1993b. Chestnut et al. (1992) and Harvey et al. (1991a) also showed positive results in sheep when consuming montmorillonite clays during AF exposure.

Following identification of AFM_1 excretion in the urine and milk of animals exposed to AFB_1, exposure of the young through nursing has been investigated. Importantly, inclusioin of 1% montmorillonite clay was able to reduce excretion of AFM_1 in the milk of dairy cows and goats by 44% and 51.9%, respectively (Harvey et al. 1991b; Smith EE et al. 1994). Reductions in the excretion of such biomarkers indicate a decrease in absorption of AFB_1 in the GI tract due to binding with the clay treatment, resulting in reduced toxin bioavailability. The AFM_1 biomarker has also been utilized in dogs and rodents to indicate efficacy of montmorillonites (Bingham et al. 2004; Sarr et al. 1995). Bingham et al. (2004) reported a reduction of 48.4% in urinary AFM_1 excretion in dogs, while Sarr et al. (1995) reported a much higher efficacy in rats (approximately 90% reduction in urinary AFM_1). Importantly, pregnant rats treated with 2 mg/kg AFB_1 showed significant maternal and developmental toxicities in their offspring; however treatment with 0.5% NS resulted in development similar to absolute controls (Mayura et al. 1998). These studies indicated that treatment of montmorillonite clay can reduce the toxicity of AFB_1 in multiple animal species, but its efficacy can differ between species due to differences in length of digestion, metabolism, dietary habits, and excretion. Importantly, based on results from short-term animal studies, it was calculated that the minimal effective dose to significantly reduce aflatoxicosis was 0.5% montmorillonite clay (Phillips 1990; Phillips et al. 1990, 1995).

Determination of efficacy from human intervention trials is currently underway at Texas A&M University. However, there is one published report of the overall efficacy in an adult population from Ghana (Wang P et al. 2008). In this trial, the minimal

effective dose, 0.5% or 3 g NS, was taken daily, divided between a breakfast, lunch, and dinner meal for three months. Effect of NS treatment on AF bioavailability was assessed by both urinary and serum biomarkers, AFM_1 and AFB_1-alb, respectively. Urinary AFM_1 levels were significantly reduced (58.7%) in the NS treated group when compared to a blinded placebo after three months of treatment. AFB_1-alb levels were significantly lower in both the high-dose and low-dose NS treatment groups when compared to the placebo at the three month mark as well. Importantly, this significant difference was lost after treatment was discontinued (Wang P et al. 2008). This is the only published study to demonstrate efficacy of a montmorillonite clay to reduce bioavailability of AF in a human population at high-risk for exposure.

1.4.5 Safety evaluation

Safety assessment of both NS and UPSN has been conducted in animal and adult human trials. Due to the structural and physical characteristics it was postulated that montmorillonites could negatively affect the utilization of essential nutrients through cation exchange and possible sequestration of certain minerals like Zn, Mn, Na, and P in the interlayer. Studies specifically designed to determine the safety of smectite clay consumption are summarized in Table 2.

Table 1. Efficacy of montmorillonite clays to protect against mycotoxins

Species	Mycotoxin levels	Clay in feed (duration)	Major effects reported	Reference
Mice	Zearalenone	400 mg/kg bw; 5 g/kg bw (48 h)	Prevented the general toxicity of ZEN.	Abbès et al. 2006
Mice	Zearalenone	400, 600 or 800 mg/kg bw (48 h)	Reduced erythrocytes, decreased the chromosomal aberration frequency and increased the number of polychromatic erythrocytes in bone-marrow cells.	Abbès et al. 2007
Rats	Aflatoxins	5g/kg (30 d)	Prevented deleterious effects of aflatoxins.	Abbès et al. 2010
Rats	Aflatoxins	0.5% (21 d)	Decreased growth inhibition in pregnant rats.	Abdel-Wahhab et al. 1998
Chickens	Aflatoxins	0.5 % (28 d)	Clay counteracted some of the toxic effects of AF in growing broiler chicks.	Abo-Norag et al. 1995
Chickens	Afl/T-2 toxin	0.5% (21 d)	No protection against T-2 toxin, variability in protection against growth inhibition	Bailey et al. 1998
Pigs	Aflatoxins	0.50%	Decreased DNA adducts in the liver and reduced tissue residues of total aflatoxins.	Beaver et al. 1990
Dogs	Aflatoxins	0.5% (48 h)	Significantly reduced the bioavailability of aflatoxins and excretion of M_1 in urine.	Bingham et al. 2004
Mink	Aflatoxins	0.5% (77 d)	Mortality was prevented.	Bonna et al. 1991
Mink	Zearalenone	0.5% (24 d)	Clay did not appreciably alter the hyperestrogenic effects.	Bursian et al. 1992
Rats (& Sheep)	Ergotamine	Rats: 2.0% (28 d)	HSCAS did not significantly protect rats or sheep from fescue toxicosis.	Chestnut et al. 1992

Table 1. Continued

Species	Mycotoxin levels	Clay in feed (duration)	Major effects reported	Reference
Pigs	Aflatoxins	0.5% (35 d)	Clay prevented hepatocellular changes normally associated with Aflatoxin consumption.	Colvin et al. 1989
Chickens	Cyclopiazonic acid	1.0% (21 d)	Clay did not significantly prevent the adverse effects of clyclopiazonic acid.	Dwyer et al. 1997
Turkeys	Aflatoxins	0.5% (21 d)	Decreased urinary excretion of aflatoxin M_1.	Edrington et al. 1996
Chickens	Aflatoxins	0.5% (3 weeks)	Improved feed intake and weight gain. Alleviated the adverse effects of AFB_1 on some serum chemistry.	Gowda et al. 2008
Lambs	Aflatoxins	2.0% (42 d)	Diminished growth inhibition and immunosuppression.	Harvey et al. 1991a
Dairy Cows	Aflatoxins	0.5%; 1.0% (28 d)	Reduction of aflatoxin M_1 in milk.	Harvey et al. 1991b
Pigs	Aflatoxins	0.5%; 2.0% (28 d)	Decreased growth inhibition; prevention of serum effects and hepatic lesions.	Harvey et al. 1994
Pigs	Aflatoxins	0.5%; 2.0% (28 d)	Diminished growth inhibition, hepatic lesions and immunosuppression.	Harvey et al. 1989
Chickens	Afl/Ochratoxin A	0.5% (21 d)	Decreased growth inhibitory effects; no effect against ochratoxin.	Huff et al. 1992
Chickens	Aflatoxin	0.35, 0.5% (21 d)	Reduced immune stimulation effects of Afl	Kececi et al. 1998
Chickens	Afl/Trichothecenes	0.5% (21 d)	Growth inhibition diminished; no effect on trichothecens.	Kubena et al. 1990b
Chickens	Aflatoxin	0.5% (28 d)	Growth inhibition diminished; decreased mortality..	Kubena et al. 1990a

Table 1. Continued

Species	Mycotoxin levels	Clay in feed (duration)	Major effects reported	Reference
Turkeys	Aflatoxins	0.5% (21 d)	Decreased mortality.	Kubena et al. 1991
Chickens	Afl/Trichothecens	0.25%; 0.37%; 0.8% (21 d)	Diminished growth inhibition; Alleviated the adverse effects of AFB_1 on serum chemistry; no effect against trichothecenes.	Kubena et al. 1993a,b
Chickens	Aflatoxin, T-2	0.25, 0.375% (21 d)	Growth inhibition diminished; relative organ weights similar to controls	Kubena et al. 1998
Chickens	Aflatoxins	1.0% (21 d)	Growth inhibition completely prevented.	Ledoux et al. 1999
Pigs	Aflatoxins	0.5% (42 d)	Diminished growth inhibition.	Lindemann et al. 1993
Chickens	Aflatoxin,	0.2% (42 d)	Increased feed intake and apparent retention of phosphorus. Prevented adverse effects to mycotoxins.	Liu et al. 2011
Chickens	Aflatoxin	0.5, 1.0% (21 d)	Growth inhibition diminished; Ameliorated feed intake and feed efficiency effects	Marquez-Marquez et al. 1995
Hydra	Aflatoxin	0.1 %; 0.3%; 0.5% (92 hr)	NS clay saved hydra from Afl-induced toxicity	Marroquin-Cardona et al. 2009
Rats	Aflatoxins	0.5% (21 d)	Significant prevention of maternal and developmental toxicity.	Mayura et al. 1998
Chickens	Aflatoxins	0.5% (28 d)	Growth inhibition diminished; gross hepatic changes prevented.	Phillips et al. 1988
Chickens	Aflatoxins	0.125%; 0.25%; 0.5% (21)	Protected against vitamin A depletion in the livers of chicks exposed to aflatoxins.	Pimpukdee et al. 2004

Table 1. Continued

Species	Mycotoxin levels	Clay in feed (duration)	Major effects reported	Reference
Rats	Fumonisin	2% (72 hr)	Significantly reduced FB_1 biomarkers	Robinson et al. 2012
Rats	Aflatoxins	0.5% (21 d)	Decreased urinary excretion of Aflatoxin metabolites (M_1 & P_1).	Sarr et al. 1995
Pigs	Aflatoxins	1.0% (42 d)	Lowered Mg and Na absorption, restoration of performance and liver function.	Schell et al. 1993 a
Pigs	Aflatoxins	0.5% (35 d)	Growth inhibitory effects reduced.	Schell et al. 1993 b
Dairy Goats	Aflatoxins	1.0%; 2.0%; 4.0% (12 d)	Reduction of aflatoxin M_1 in milk.	Smith et al. 1994
Human	Aflatoxin	1.5 g/day; 3 g/day (3 mo)	Significantly reduced AFM_1 biomarker in urine and AFB_1-albumin biomarker in serum.	Wang P et al. 2008
Rats	Aflatoxins	0.1%; 1% (8 wks)	Partial protection against lesions in the liver.	Voss et al. 1993
Chickens	Aflatoxins	0.1%; 0.2% (21 d)	Clay effectively alleviated the negative effect of AFB_1 on growth performance and liver damage.	Zhao et al. 2010

HSCAS-hydrated sodium calcium aluminosilicate, NS-NovaSil.
Adapted from Johnson 2010.

Initial trials in chicks fed control diet with 0.5 or 1% montmorillonite clay showed no significant differences in weight gain or feed efficiency when compared to controls (Chung et al. 1990). The same was true for riboflavin, liver vitamin A concentrations, tibia weight, and manganese (Mn) concentrations (Chung et al. 1990). However, this same study did show a dose-dependent decrease in tibia zinc (Zn) concentration, with the 0.5 and 1% clay groups having 5 and 14% less Zn than the controls, respectively. To evaluate use of phosphorous (P) tibia ash weight and bone-breaking force were analyzed in the presence of montmorillonite clay chickens fed diets containing P supplementation and 0.5 or 1% clay (Chung and Baker 1990). Results showed no significant differences between control groups supplemented with P and those receiving P and clay, indicating that montmorillonite clay does not impair the utilization of inorganic P (Chung and Baker 1990). Similarly, a form of montmorillonite clay given to broiler chicks at 0.5% w/w with nutrient deficient diets did not adversely affect percentage of tibia ash or Ca and P concentrations (Southern et al. 1994). A sub-chronic treatment trial in Sprague-Dawley rats was conducted with NS inclusion ranging from 0.25-2% w/w feed for 6 months, following which the authors determined no significant differences in total body weight gain, feed conversion efficiency, histological abnormalities in target organs, or serum vitamin and nutrient levels (Afriyie-Gyawu et al. 2005). The authors thus concluded that ingestion of up to 2% NS was apparently safe in a sub-chronic treatment protocol. Similarly, UPSN was assessed for safety in Sprague-Dawley rats; however treatment in this study was reduced to a 3-month exposure time period (Marroquín-Cardona et al. 2011). Results from that research

indicated that, the serum Na, Na/K ratio, and Ca in UPSN treated groups were significantly higher than controls; however it was determined that the values were still within clinical ranges for these animals. Interestingly, this study also reported a significant increase in vitamin E values at both levels of UPSN inclusion. Other significant differences observed by Marroquin-Cardona et al. (2011) did not follow a dose-dependent relationship or were not consistent across both sexes. Therefore, the authors concluded that UPSN treatment at concentrations up to 2% resulted in no overt toxicity. Furthermore, Wiles et al. (2004) examined the gastrointestinal bioavailability of metals in pregnant rats following exposure to NS and concluded that the clay did not significantly leach or prevent absorption of the 11 metals analyzed.

Results of Phase I and II clinical trials with NS clay suggested that ingestion of up to 3 g/day of NS in adults is safe for a 3 month period. There were no significant differences in clinical chemistry parameters, hematology, blood electrolytes, vitamins A and E, or serum Ca, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Se, Si, and Zn (Afriyie-Gyawu et al. 2008a, 2008b; Wang JS et al. 2005). The greatest hazard associated with acute ingestion of similar clay materials is intestinal obstruction. There have also been reports of increased fecal elimination of K and Fe resulting in clinical hypokalemia with Fe-deficient anemia following chronic consumption of Al-silicate clays (Willhite et al. 2012). However, it is important to note that these adverse event reports arose from studies in which adults consumed large quantities (0.5-1.0 kg/day) of various types of Al-silicate clays that were not assessed for metals or environmental contaminants (Reviewed by Willhite et al. 2012). Based on detailed studies conducted in animals and

humans for NS and UPSN, it was determined that ingestion of UPSN at levels efficacious for reducing AFB_1 biomarkers would be reasonably safe. Results from mineralogical analyses of UPSN and NS indicated similar structural, morphological, and chemical characteristics (Marroquín-Cardona et al. 2011); therefore the two materials are thought to possess similar safety profiles. Importantly, Ca-montmorillonites, labeled as hydrated sodium calcium aluminosilicate (HSCAS) in the past literature, have been given GRAS (generally recognized as safe) status by the U.S. FDA for inclusion not exceeding a level of 2% w/w in feeds.

Table 2. Safety studies with montmorillonite clays

Species	Clay in feed (duration)	Major effects reported	Reference
Chickens	0.5%; 1.0% (14 d)	NS did not impair phytate or inorganic phosphorous utilization.	Chung & Baker 1990
Chickens	0.5% (19 d)	Did not affect growth performance or tibial mineral concentrations of chicks.	Southern et al. 1994
Chickens	0.5%; 1.0% (14 d)	NS did not impair utilization of riboflavin, vitamin A, or Mn; slight reduction of Zn.	Chung et al. 1990
Human	1.5 g/day; 3 g/day (3 mo)	No significant effects in vitamins A & E and micronutrients, except for Strontium.	Afriyie-Gyawu et al. 2008
Rats	0.25%; 2.0% (3 mo)	Increased serum Ca, Na, Vit. E. Reduced Zn in males %2 clay. Reduced serum K in males of clay groups.	Marroquin-Cardona et al. 2011
Human	1.5 g; 3 g (2 wks)	Mild GI effects, not significantly different. No difference in hematology, electrolytes, liver and kidney function.	Wang JS et al. 2005
Human	1.5 g/day; 3 g/day (3 mo)	Moderate effects, though not significant. No significant difference in hematology, electrolytes, liver and kidney function.	Afriyie-Gyawu et al. 2008
Rats	0.25%; 0.5%; 1.0%; 2.0% (6 mo)	No adverse effects including vitamin utilization.	Afriyie-Gyawu et al. 2005
Rats	2.0% (16d)	In pregnant rats, Rb was reduced in groups with clay. Neither NSP nor SWY-2 influenced mineral intake.	Wiles et al. 2004
Humans (children)	6 g/day; 12 g/day (3d)	Adverse events were not significantly different from placebo. Major complaints were vomiting, fever.	Dupont et al. 2009
Humans (children)	1.5 g/day (3 d)	No adverse events were reported.	Madkour et al. 1993

1.5 Research objectives

Mycotoxin contamination of staple crops is seemingly inextricable and exposure is inevitable in developing countries, where food insecurity results in consumption of lower quality grains (Williams et al. 2004). Assessment of linear biomarkers of exposure to mycotoxins in diverse human populations has shed light recently on the health burdens from AF and FB exposure in Western Africa. It is hypothesized that exposure of vulnerable, high risk populations to both mycotoxins could significantly impact health and disease outcomes. However, field-practical strategies to reduce mycotoxin exposures have proven difficult to maintain in rural communities of Africa. Thus, intervention strategies that are economically feasible, culturally acceptable, easily maintained, and can be implemented in communities at the highest risk for exposure during mycotoxin outbreaks are critically needed. Utilization of a natural product, like calcium montmorillonite clays, as a toxin enterosorbent in food could act as such an intervention. Work with multiple animal species has indicated a high efficacy of NovaSil in protecting animals from AF-induced health effects. However, minimal effective doses have not been delineated in humans and safety assessment is still ongoing. Recent work has indicated that NovaSil clay also binds FB in the interlayer to a lesser extent than AF (Robinson et al. 2012; Brown et al. 2012). Consequently, it is possible that NovaSil (and similar clays) may be used to mitigate (and reclaim) food-stuffs contaminated with both toxins. The work described in this dissertation focuses on utilizing mycotoxin biomarkers to establish exposures, efficacy, and safety of refined NovaSil clay (UPSN) in a high-risk community in Ghana. The work also aims to delineate effecitve dose

delivery methods and the ability of UPSN to alter the bioavailability of mixtures of AFB_1 and FB_1. The specific aims are fourfold:

1) to assess the average AF exposure across different regions of Ghana in relation to various sociodemographic, cultural, and health factors through the use of a comprehensive questionnaire and short-term analysis of AFM_1 in the urine.

2) to evaluate the effectiveness of UPSN in a cross-over clinical intervention trial with treatment provided in the diets of Ghanaian participants to determine the ability of a short-term biomarker (AFM_1) from urine as to be used as an early indicator of intervention efficacy.

3) to investigate the safety of UPSN during a Phase I clinical trial in children (ages 3-9) and provide initial evidence for aflatoxin exposure and UPSN efficacy.

4) to confirm the ability of UPSN to decrease the bioavailability of both AFB_1 and FB_1 in the rat using short-term and long-term biomarkers of exposure.

2. EPIDEMIOLOGICAL SURVEY OF AFLATOXIN EXPOSURE ACROSS REGIONS OF GHANA

2.1 Introduction

Previous work in Ghana has indicated that adults residing in the Ejura-Sekyedumase district of the Ashanti region are chronically exposed to high levels of AFs via the diet (Jolly et al. 2006; Wang P et al. 2008). During the screening process for a three-month intervention trial, 100% of volunteers from this community tested positive for AFB_1-alb in their serum. However, AF exposure in the rest of the country is not well established and may vary across socioeconomic classes and/or between regions based on differences in dietary intake. Previously, Jolly et al. (2006) observed significant correlations between AFB_1-alb levels and education level, occupation, ethnic group, village of residence, number of individuals in the household, and number of children during a survey in the Ejura district. Recent risk assessment of AF burden in African countries has indicated that Ghanaian populations are at highest risk for exposure when compared to Kenya, Gambia, Botswana, Benin, and Tanzania (Shephard et al. 2008). During this risk assessment it was postulated that an average 60 kg adult in Ghana will consume as much as 1000 g of maize based foods per day, resulting in a total daily exposure of 850 ng/kg body weight. Further assessment indicated that HCC cases per 100,000 population due to AF exposure in Ghana may be as high as 70. Thus, potential factors that could increase the relative risk for exposure in Ghana need to be determined. Identification of these risk factors would indicate areas (and communities) where intervention is most needed and would prove the most efficacious.

2.2 Materials and methods

2.2.1 Materials

High Performance Liquid Chromatography (HPLC) grade methanol and ethanol, as well as reagent grade pH buffers (4.0, 7.0, and 10.0) were purchased from VWR (Atlanta, GA). AFM_1 analytical standard was purchased from Sigma-Aldrich (Saint Louis, MO). AFM_1 stock concentrations were verified by UV-visible spectrophotometry at a wavelength of 352 nm and molar absorptivity of ε=18,815 $M^{-1}cm^{-1}$ (Shimadzu, Kyoto, Japan). AflatTest immunoaffinity columns were purchased from VICAM (Watertown, MA, USA). Ultrapure deionized water (18.2 MΩ) was generated within the laboratory using an Elga™ automated filtration system (Woodridge, IL).

2.2.2 Participant recruitment, questionnaire, and data collection

Cross-sectional field surveys of demographic, food handling, consumption, health status, health history, and lifestyle choices were conducted in six different regions of Ghana (Ashanti, Brong Ahafo, Central, Eastern, Greater Accra, and Northern) by trained study monitors, June through August 2004. Only one adult participant from each household could participate in the survey and study monitors explained the purpose of the study to prospective participants prior to requesting consent. Consent and surveys were read to participants in their local language. A total of 801 participants completed the full survey and provided one urine sample to be analyzed for AFM_1 levels. The number of participants from each region are as follows: Ashanti: 298, Brong Ahafo: 37, Central: 114, Eastern: 6, Greater Accra: 285, and Northern: 19. Variations in the number of participants recruited were calculated prior to initiation of the study and were based

on population size for each region. A greater number of recruits were desired from those regions with the largest portion of Ghana's population, i.e. Accra and Ashanti.

2.2.3 Determination of AFM_1 in urine

Analysis of urinary AFM_1 levels followed methods reported by Groopman et al. (1992b) and Sarr et al. (1995). Urine samples were centrifuged at 2300 rpm, and 5.0 ml of supernatant was collected, acidified with 0.5 ml of 1.0 M ammonium formate (pH 4.5) and diluted with water to a total volume of 10.0 ml. Samples were then loaded onto a 3 ml preparative Aflatest® WB immunoaffinity column (VICAM, Watertown, MA, USA) at a flow rate of 1 ml/min. Following washing of the column, the AF fraction was eluted with 2 ml of 80% methanol, dried under N_2 and re-suspended in 200 µl of a 1:1 solution of methanol:20 mM ammonium formate. Samples were analyzed using a Shimadzu HPLC system (Waters, Watertown, MA, USA) with fluorescence detection capabilities. A 250 x 4.6 mm LiCrospher RP-18 column with pore size 100 Å and particle size 5 µm (Alltech Associates, Deerfield, IL, USA) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol buffered with 20 mM ammonium formate (pH 3.0) in water. Isocratic elution of the mobile phase for 20 min at a rate of 1 ml/min allowed for proper chromatographic separation. External AFM_1 standards were prepared weekly and injected following every 5 injections of samples. The limit of detection for this method was 4.8 pg for AFM_1. Urinary AFM_1 concentrations were expressed as pg/mg creatinine to correct for variations in urine dilution among samples. Urinary creatinine concentrations were measured by a Selectra E auto-analyzer (Vital Scientific, The Netherlands).

2.2.4 Statistical analysis

Statistical analyses were conducted by categorizing the participants into one of three groups based on the AFM_1 level. The first group had an AFM_1 level of 0.5 ppb or less, the second group had an AFM_1 level greater than 0.5 ppb and less than or equal to the median AFM_1 level of 43.2 ppb, and the third group had an AFM_1 level of more than 43.2 ppb. The analyses were then conducted separately for each of the three categories. Categorical variables were reported as counts and percentages. The chi-squared or Fisher's exact tests were used to compare categorical variables. Variables with p-value $\leq$ 0.10 in a bivariate model were included in the multivariable model. Odds ratios (OR) and the p-values for testing the significance of the variables in the model were obtained. All tests with a p-value less than or equal to 5% were deemed statistically significant and all analyses were done using SAS software version 9.2 (SAS Institute, Cary, NC, USA).

2.3 Results

2.3.1 Aflatoxin M_1 concentration in urine

Urinary AFM_1 was detected in 40.9% of the 801 samples tested. The median, mean, and range of AFM_1 levels are presented in Table 3. Approximately one sixth of the population was above the mean level of 276 pg AFM_1/mg creatinine. Concentration of AFM_1 excretion ranged from 0 to 9,532 pg AFM_1/mg creatinine and the distribution is represented in Figure 6. Estimates of AFB_1 exposure levels were calculated from the median, 75^{th} and 90^{th} percentiles, and maximum AFM_1 values (Table 4). Briefly, it was assumed that the average adult in the study would excrete 1500 ml of urine per day and 2% of the total AFB_1 consumed would be excreted as AFM_1 in the urine (Zhu et al.

1987). The portion of the population above the 90th percentile was exposed to approximately greater than 43 μg of AFB_1 per day.

2.3.2 Sociodemographic characteristics

Table 5 shows the demographic and socio-economic characteristics of the 801 participants by AFM_1 level. Males and females were almost equally represented in the study, 49.7% and 50.3% respectively. However, a significant majority, 71.3%, of the heads of household were male. The majority of heads of households, 52.3%, were 50 years or younger while the majority of the respondents, 53.8%, were 30 years or younger. A vast majority of participants, 86.8%, received a formal education and graduated. Most of them, 68.1%, went up to secondary school. Employment was high among the participants, 72.2%, and most of them neither drank nor smoked, 67.7% and 96.8% respectively. However, none of these parameters showed a significant correlation to AFM_1 excretion.

Table 6 represents the levels of AFM_1 excretion by region of residence. Most of the participants, 76.5%, were from the Ashanti and Greater Accra regions. This is reflective of the percent of HCC mortalities for each region. It is interesting to note that this does not correlate with population size. The Ashanti region is the most populated region accounting for 19.4% of the total population, followed by the Accra region, Eastern/Western, Northern, and Brong Ahafo. The Central region is the least populated based on the nation's 2010 consensus (Ghana Statistical Service 2013). The region the participants came from had a significant association ($p = 0.05$) with their AFM_1 levels. The Northern region had the highest percentage of its participants in the high AF group,

however there was only one more person reported than in the other two AFM_1 levels. The Central region had 25.4% of its population within the high AF group followed by the Greater Accra region 23.5%. The Ashanti and Eastern groups had similar percentages within the high AF group, while the Brong Ahafo region had the lowest percentage (10.8%) of its participants in that group.

2.3.3 Health factors

Table 7 shows the health factors of the participants by AFM_1 level. Most of the participants, 63.6%, reported being very healthy. To take care of their health care needs, most (59.7%) used orthodox means at government hospitals (54.6%) rather than use traditional means or self-medication. The majority had not been hospitalized recently, 67.4%. When asked about their health condition, more than 80% of household members reported not having any of the health conditions present on the questionnaire: quick yellowing of skin, yellowing of eyes, dark brown urine, painful vomiting, and sore or swollen stomach. Most of the participants, more than 60%, also reported that they did not know whether their illness was related to foods such as peanuts, peanut products, maize, or maize products.

Table 3. Descriptive statistics of AFM_1 levels

AFM_1 levels (pg/mg creatinine)	
Number positive	328
Mean ± SD	276 ± 883
Median	43
Range	0-9,532
Percentiles	
25	15
50	43
75	136

2.3.4 Food consumption and preparation practices

Table 8 shows the food consumption of main products most commonly associated with AF exposure by AFM_1 level. On a weekly basis, 41, 93.3, and 26.8% of households consumed groundnut or groundnut products, maize or maize products, and millet or millet products, respectively, no more than once. Maize or maize products were consumed everyday by 61.8% of families. Of all the foods consumed, only maize or maize products had a significant association ($p = 0.02$) with AFM_1 levels. A great majority of participants (89.5%) could identify spoilt food. More than half of participants (50.2%) reported that a handful of their food was spoilt while a little less than half (47.6%) reported that a bucketful of their food was spoilt. Only a small portion, (2.27%) reported more than a bucketful of food spoilage.

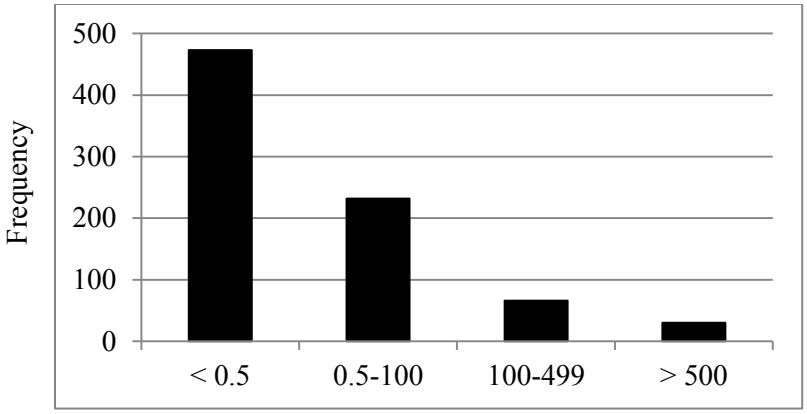


Figure 6. Distribution of detectable aflatoxin M_1 levels

Table 4. Estimated AFB_1 exposure calculated from AFM_1 excretion levels

	μg AFB_1/day
Median (exposed)	2.5
75th Percentile	9.9
90th Percentile	43.1
Maximum	614.1

Table 9 shows the consumption behavior of the study participants by AFM_1 level. In general, most of the participants took the right actions with their grains or nuts. For example, 93.8% washed their grains or nuts before cooking and 97.7% cleaned their utensils before grinding grains or nuts. Also, 77.5% discarded discolored grains or nuts and only 36% used the discolored grains or nuts to feed animals. Participants also showed a lot of caution in buying what they consumed because 77.4% examined the grains or nuts for spoilage before buying them. None of the consumption behaviors had a significant association the AFM_1 levels.

Table 5: Demographic and Socio-economic Information by AFM_1 level

Variable	All n = 801	$AFM_1 \leq$ 0.5 n = 473	$0.5 < AFM_1 \leq$ 43.2 n = 163	$AFM_1 >$ 43.2 n = 165	pvalue
Gender of respondent					
Male	378(49.7)	221(48.6)	67(45.3)	90(57.3)	0.08
Female	382(50.3)	234(51.4)	81(54.7)	67(42.7)	
Age of respondent					
Age ≤ 30	431(53.8)	244(51.6)	99(60.7)	88(53.3)	0.13
Age > 30	370(46.2)	229(48.4)	64(39.3)	77(46.7)	
Gender of head of household					
Male	528(71.3)	313(70.7)	97(66.4)	118(77.6)	0.09
Female	213(28.7)	130(29.4)	49(33.6)	34(22.4)	
Age of head of household					
Age ≤ 50	383((52.3)	234(53.3)	74(51.8)	75(50.0)	0.77
Age > 50	349(47.7)	205(46.7)	69(48.3)	75(50.0)	
Formal Education					
Yes	651(86.8)	393(87.7)	121(83.5)	136(87.2)	0.55
No	99(13.2)	55(12.3)	24(16.6)	20(12.8)	
Level of Education					
Primary	49(7.45)	31(7.8)	9(7.2)	9(6.6)	
Secondary	448(68.1)	258(65.0)	91(72.8)	99(72.8)	0.26
Tertiary	142(21.5)	93(23.4)	22(17.6)	27(19.9)	
Other	19(2.9)	15(3.8)	3(2.4)	1(0.74)	
Graduated					
Yes	568(86.7)	345(87.3)	106(85.5)	117(86.0)	0.94
No	87(13.3)	50(12.7)	18(14.5)	19(14.0)	
Employed					
Yes	539(72.2)	317(71.1)	90(72.0)	114(74.0)	0.72
No	208(27.8)	129(28.9)	35(28.0)	40(26.0)	
Drinking					
Yes	244(32.4)	152(33.7)	38(30.4)	51(32.7)	0.42
No	510(67.6)	299(66.3)	87(69.6)	105(67.3)	
Smoking					
Yes	24(3.2)	14(3.1)	5(4.0)	5(3.2)	0.97
No	730(96.8)	439(96.9)	119(96.0)	150(96.8)	

Data are presented as total number (n) and (%). * Statistical significance

Table 6. AFM_1 concentration by region

	Total	$AFM_1 \leq 0.5$	$0.5 < AFM_1 \leq 43.2$	$AFM_1 > 43.2$	
	n = 801	n = 473	n = 163	n = 165	pvalue
					0.05*
Ashanti	298	182(61.1)	67(22.5)	49(16.4)	
Brong Ahafo	37	24(64.9)	9(24.3)	4(10.8)	
Central	114	70(61.4)	15(13.2)	29(25.4)	
Eastern	6	5(83.3)	0(0.0)	1(16.7)	
Greater Accra	285	168(58.9)	50(17.5)	67(23.5)	
Northern	19	6(31.6)	6(31.6)	7(36.8)	

Data is represented as total number and (percent by region). * statistical significance

2.4 Discussion

AF exposure in Ghana is an ongoing public health problem, which can contribute to the high morbidity and mortality in the country. However, until recently studies assessing the AF exposure pattern in Ghana have been lacking, with most occurring in a specified region (Ashanti) where chronic exposure is known to occur (Jolly et al. 2006; Wang P et al. 2008). Although efforts to control and reduce the AF exposure in Ghana have already been initiated more demographic information is needed. Demographics, socio-economic status, and food consumption practices in relation to AFB_1 exposure would provide useful information about steps in the food production/consumption chain that are most likely contributing to exposure. This is the first study in Ghana to assess exposure across multiple regions of the country. Although total numbers of participants were highly variable between the regions, by classifying exposure status as low, medium, or high we were able to make comparisons between percentages of participants in a specific classification across regions. Through this method we are able to conclude that the Greater Accra and Central regions have very similar exposure patterns, which

Table 7: Health Factors by M_1 level

Variable	All n = 801	$AFM_1 \leq 0.5$ n = 473	$0.5 < AFM_1 \leq 43.2$ n = 163	$AFM_1 > 43.2$ n = 165	pvalue
Status of Health					0.09
Very Healthy	473(63.6)	279(62.7)	87(60.0)	107(69.5)	
Average	235(31.6)	138(31.0)	53(36.6)	44(28.6)	
Poor	36(4.8)	28(6.3)	5(3.5)	3(2.0)	
Type of Healthcare					0.19
Orthodox	446(59.7)	275(61.4)	86(59.7)	85(54.8)	
Traditional	17(2.3)	9(2.0)	4(2.8)	4(2.6)	
Spiritual	2(0.3)	1(0.2)	0(0.0)	1(0.7)	
Self Medication	142(19.0)	84(18.8)	34(23.6)	24(15.5)	
Combination	133(17.8)	75(16.7)	20(13.9)	38(24.5)	
None	6(0.8)	4(0.9)	0(0.0)	2(1.3)	
Other	1(0.1)	0(0.0)	0(0.0)	1(0.7)	
Type of Hospital					0.45
Government	375(54.6)	228(55.2)	71(54.6)	76(52.8)	
Private	137(19.9)	86(20.8)	21(16.2)	30(20.8)	
Both/Clinic	97(14.1)	52(12.6)	20(15.4)	25(17.4)	
Own Home	71(10.3)	44(10.7)	17(13.1)	10(6.9)	
Family	7(1.0)	3(1.7)	1(1.8)	3(2.1)	
Been Hospitalized					0.08
Yes	238(32.7)	156(35.8)	41(29.3)	41(26.8)	
No	491(67.4)	280(64.2)	99(70.7)	112(73.2)	
Health Condition of Household Members					
Quick Yellowing of skin					0.39
Yes	62(8.2)	41(9.1)	8(5.4)	13(8.3)	
No	693(91.8)	410(90.9)	139(94.6)	144(91.7)	
Yellowing of eyes					0.47
Yes	116(15.4)	72(16.1)	18(12.2)	26(16.7)	
No	636(84.6)	376(83.9)	130(87.8)	130(83.3)	

Table 7. Continued

	All	$AFM_1 \leq 0.5$	$0.5 < AFM_1 \leq 43.2$	$AFM_1 > 43.2$	pvalue
Dark brown urine					0.57
Yes	89(11.8)	58(12.9)	15(10.2)	16(10.2)	
No	665(88.2)	392(87.1)	132(89.8)	141(89.8)	
Swollen Legs					0.04*
Yes	56(7.4)	41(9.1)	10(6.8)	5(3.2)	
No	700(92.6)	410(90.9)	137(93.2)	153(96.8)	
Swollen Toes					0.20
Yes	30(4.0)	23(5.1)	3(2.0)	4(2.5)	
No	725(96.0)	427(94.9)	144(98.0)	154(97.5)	
Swollen Arms					0.31
Yes	15(2.0)	12(2.7)	1(0.7)	2(1.3)	
No	740(98.0)	439(97.3)	146(99.3)	155(98.7)	
Swollen Fingers					0.74
Yes	17(2.3)	12(2.7)	2(1.4)	3(1.9)	
No	736(97.7)	437(97.3)	145(98.6)	154(98.1)	
Painfull Vomiting					0.63
Yes	71(9.4)	39(8.7)	16(10.9)	16(10.2)	
No	683(90.6)	411(91.3)	131(89.1)	141(89.8)	
Sore or Swollen Stomach					0.26
Yes	87(11.5)	45(10.0)	21(14.2)	21(13.5)	
No	667(88.5)	405(90.0)	127(85.8)	135(86.5)	
Illness Relating to Food					
Peanuts					0.18
Yes	69(15.5)	46(16.6)	15(17.7)	8(9.6)	
No	104(23.4)	61(22.0)	16(18.8)	27(32.5)	
Don't Know	272(61.1)	170(61.4)	54(63.5)	48(57.8)	

Table 7. Continued

	All	$AFM_1 \leq 0.5$	$0.5 < AFM_1 \leq 43.2$	$AFM_1 > 43.2$	pvalue
Peanuts Products					0.17
Yes	69(15.5)	46(16.7)	15(17.7)	8(9.5)	
No	102(23.0)	59(21.5)	16(18.8)	27(32.1)	
Don't Know	273(61.5)	170(61.8)	54(63.5)	49(58.3)	
Maize					0.84
Yes	27(6.1)	16(5.8)	6(7.1)	5(6.0)	
No	120(27.0)	74(26.9)	20(23.5)	26(31.0)	
Don't Know	297(66.9)	185(67.3)	59(69.4)	53(63.1)	
Maize Products					
Yes	27(6.1)	17(6.2)	5(5.9)	5(6.0)	0.79
No	121(27.2)	74(26.8)	20(23.5)	27(32.1)	
Don't Know	297(66.7)	185(67.0)	60(70.6)	52(61.9)	

Data is represented as total number and (%). * Statistical significance

happen to be some of the highest. Both Accra and Central regions are located on the coast and thus may engage in similar dietary habits more than other areas. Interestingly, in the Central region over half of its population, 52.9%, is located in rural areas while in Accra 90.5% of its population is in urbanized areas (Ghana statistical service 2013). However, they do have the two highest population densities of all the regions, albeit Accra is more densely populated than any other region, having 1,235.8 people per square kilometer of land. The Northern region had the highest percentage (68.4%) of its population test positive for AFM_1 in the urine, which could be indicative of the heightened presence of agriculture in the area. The northern areas of the country tend to be more rural and are where the countries crops are primarily produced. Interestingly, this region is predominantly Islamic with three out of every five households indicating Islam as their religious affiliation (Ghana statistical service 2013).

Table 8: Percent Food Consumption by AFM_1 level

Variable	All n = 801	AFM_1<0.5 n = 473	0.5< AFM_1 ≤ 43.2 n = 163	AFM_1> 43.2 n = 165	pvalue
Frequency of food consumption by household/week					
Groundnut/Groundnut products					0.24
One or less	53.1	53.2	54.4	51.2	
Two to three	29.1	29.7	32	24.4	
Everyday	11.9	11.3	10.2	15.4	
Never	6	5.8	3.4	9	
Maize/Maize products					0.02*
One time or less	6.4	6.9	6.1	5.1	
Two to three	31.5	35.3	27.2	24.4	
Everyday	61.8	57.8	66	69.2	
Never	0.4	0	0.7	1.3	
Millet/Millet products					0.52
One time or less	34.6	34.5	34	35.3	
Two to three	18	16.9	21.5	18	
Everyday	8.8	7.9	7.6	12.2	
Never	38.6	40.6	36.8	34.6	

In contrast to a previous report from the Ashanti region of Ghana (Jolly et al. 2006) there were no significant associations with demographics or socio-economic factors and AF exposure. In that study the authors concluded that there was a correlation with education level, ethnic group, and number of individuals in a household and AFB_1-alb. The lack of associations described here could be caused by the variability in the AFM_1 biomarker and its short half-life, thus indicating exposure within 24 to 48 hr of sampling. The AFB_1-alb marker has a longer half-life, able to reach a plateau, which makes it more indicative of a person's exposure over longer time periods. Therefore, the

AFB_1-alb adduct may be a more accurate indicator of associations between biomarker levels and chronic social conditions, like demographics and socio-economic status. It is important to note however, that other work in Malaysia did not find associations between education, number of individual households, and other demographic factors with AFB_1-alb levels (Leong et al. 2012). Thus, more work needs to be conducted to conclude whether the correlations observed by Jolly et al. (2006) are maintained across other countries and ethnic groups, or if they are specific to the particular community in the Ashanti region. Furthermore, the present work here would indicate that exposure is consistent across all demographic and socio-economic classes. As a result, interventions should target populations in specific areas of the country and be applicable to people across all ages, genders, economic, and educational backgrounds.

Health indicators did not show an association with AFM_1 levels. This included reports of sickness from consumption of AF-prone foods. Calculations of AFB_1 ingestion from AFM_1 levels would indicate that exposure during the time of sampling was not high enough to induce typical symptoms of acute exposure such as vomiting and other gastrointestinal irritation. The time of sampling could have influenced reports of illness and rates of AFM_1 excretion. Ghana has its highest rainfall from April to July and AF exposure patterns have been associated with climatic changes in Africa, with higher biomarker levels observed during dry seasons compared to rainy seasons (Turner et al. 2000; Wild et al. 2000). Consequently, participants could have been exposed to lower levels of AFs during the study period than at other times during the year.

Table 9: Food processing practices by M_1 level

Variable	All n = 801	$AFM_1 \leq 0.5$ n = 473	$0.5 < AFM_1 \leq 43.2$ n = 163	$AFM_1 > 43.2$ n = 165	pvalue
Actions taken with grain/nuts					
Sort before cooking					0.71
No, no future plan	59(9.6)	37(10.1)	13(10.6)	9(7.1)	
No, have future plan	27(4.4)	20(5.4)	3(2.4)	4(3.2)	
No, planning soon	32(5.2)	16(4.4)	7(5.7)	9(7.1)	
Yes, last time	82(13.3)	46(12.5)	17(13.8)	19(15.1)	
Yes, all the time	417(67.6)	249(67.7)	83(67.5)	85(67.5)	
Wash before cooking					0.22
No, no future plan	19(3.1)	7(1.9)	5(4.1)	7(5.7)	
No, have future plan	13(2.1)	10(2.7)	2(1.7)	1(0.8)	
No, planning soon	6(1.0)	2(0.6)	2(1.7)	2(1.6)	
Yes, last time	52(8.5)	34(9.3)	7(5.8)	11(8.9)	
Yes, all the time	520(85.3)	313(85.5)	105(86.8)	102(82.9)	
Grill before cooking					0.50
No, no future plan	219(38.4)	127(37.0)	50(44.3)	42(36.5)	
No, have future plan	63(11.0)	35(10.2)	15(13.3)	13(11.3)	
No, planning soon	24(4.2)	13(3.8)	3(2.7)	8(7.0)	
Yes, last time	70(12.3)	44(12.8)	10(8.9)	16(13.9)	
Yes, all the time	195(34.2)	124(36.2)	35(31.0)	36(31.3)	
Clean utensils before grinding					0.57
No, no future plan	9(1.5)	6(1.6)	2(1.7)	1(0.8)	
No, have future plan	4(0.7)	3(0.8)	1(0.8)	0(0.0)	
No, planning soon	1(0.2)	1(0.3)	0(0.0)	0(0.0)	
Yes, last time	64(10.5)	36(9.9)	9(7.4)	19(15.6)	
Yes, all the time	530(87.2)	319(87.4)	109(90.1)	102(83.6)	

Table 9. Continued

	All	$AFM_1 \leq 0.5$	$0.5 < AFM_1 \leq 43.2$	$AFM_1 > 43.2$	pvalue
Store in dry place					0.62
No, no future plan	25(4.2)	18(5.0)	5(4.2)	2(1.7)	
No, have future plan	6(1.0)	5(1.4)	1(0.8)	0(0.0)	
No, planning soon	4(0.7)	4(1.1)	0(0.0)	0(0.0)	
Yes, last time	58(9.7)	34(9.4)	14(11.7)	10(8.5)	
Yes, all the time	508(84.5)	302(83.2)	100(83.3)	106(89.3)	
Discard discolored pieces					0.36
No, no future plan	105(17.3)	62(17.0)	21(17.5)	22(18.2)	
No, have future plan	16(2.6)	9(2.5)	3(2.5)	4(3.3)	
No, planning soon	15(2.5)	10(2.7)	0(0.0)	5(4.1)	
Yes, last time	82(13.5)	46(12.6)	14(11.7)	22(18.2)	
Yes, all the time	388(64.0)	238(65.2)	82(68.3)	68(56.2)	
Use discolored pieces for oil					0.66
No, no future plan	570(94.1)	342(94.0)	117(96.7)	111(91.7)	
No, have future plan	7(1.2)	5(1.4)	0(0.0)	2(1.7)	
No, planning soon	4(0.7)	2(0.6)	1(0.8)	1(0.8)	
Yes, last time	7(1.2)	3(0.8)	1(0.8)	3(2.5)	
Yes, all the time	18(3.0)	12(3.3)	2(1.7)	4(3.3)	
Use discolored pieces for animal feed					0.87
No, no future plan	350(58.0)	206(56.9)	75(62.5)	69(57.0)	
No, have future plan	21(3.5)	14(3.9)	4(3.3)	3(2.5)	
No, planning soon	15(2.5)	9(2.5)	3(2.5)	3(2.5)	
Yes, last time	85(14.1)	48(13.3)	15(12.5)	22(18.2)	
Yes, all the time	132(21.9)	85(23.5	23(19.2)	24(19.8)	
Use discolored pieces as seeds					0.06
No, no future plan	546(91.0)	332(92.5)	112(92.6)	102(85.0)	
No, have future plan	14(2.3)	8(2.2)	0(0.0)	6(5.0)	

Table 9. Continued

	All	$AFM_1 \leq 0.5$	$0.5 < AFM_1 \leq 43.2$	$AFM_1 > 43.2$	pvalue
No, planning soon	2(0.3)	1(0.3)	0(0.0)	1(0.8)	0.06
Yes, last time	9(1.5)	4(1.1)	1(0.8)	4(3.3)	
Yes, all the time	29(4.8)	14(3.9)	8(6.6)	7(5.8)	
Examine for spoilage before buying					0.87
No, no future plan	92(15.3)	52(14.4)	21(17.7)	19(15.6)	
No, have future plan	40(6.7)	26(7.2)	7(5.9)	7(5.7)	
No, planning soon	22(3.7)	11(3.1)	4(3.4)	7(5.7)	
Yes, last time	105(17.5)	60(16.7)	21(17.7)	24(19.7)	
Yes, all the time	342(56.9)	211(58.6)	66(55.5)	65(53.3)	
Inspect processed products before buying					0.91
No, no future plan	118(16.7)	73(17.2)	23(17.0)	22(15.2)	
No, have future plan	57(8.1)	34(8.0)	14(10.4)	9(6.2)	
No, planning soon	35(5.0)	23(5.4)	7(5.2)	5(3.5)	
Yes, last time	102(14.5)	60(14.1)	19(14.1)	23(15.9)	
Yes, all the time	393(55.7)	235(55.3)	72(53.3)	86(59.3)	
Consider source of processed products before buying					0.13
No, no future plan	253(36.0)	145(34.4)	55(40.4)	53(36.8)	
No, have future plan	132(18.8)	87(20.6)	28(20.6)	17(11.8)	
No, planning soon	31(4.4)	22(5.2)	4(2.9)	5(3.5)	
Yes, last time	46(6.6)	25(5.9)	6(4.4)	15(10.4)	
Yes, all the time	240(34.2)	143(33.9)	43(31.6)	54(37.5)	

Data appear as total number and (%).

Maize was the food commodity with the highest consumption. Only 0.4% of the participants answered "never" when asked how often they consume maize-based products on a weekly basis. Maize was also the only food that was associated with AFM_1 excretion. Therefore, future intervention work should focus on treatment of maize based products, or populations with the highest maize consumption. Importantly, this work indicates that the majority of the population in Ghana can identify spoiled grains or nuts and discard those which are discolored. Large portions of the population also store their grain appropriately (94.2%), sort (80.9%), and wash foods before cooking (93.8%). Thus, educational and grain processing interventions are likely to provide little relief of AF exposure in this population. More comprehensive analysis with a larger cohort and AFB_1-alb levels should be conducted across Ghana to further assess exposure patterns and possible indicators of risk. Currently, research indicates that exposure in Ghana is endemic and has been documented in several studies to be chronically affecting the population over the past ten years (Table 10). Intervention strategies that are safe, efficacious, and sustainable in individuals across all ages, education levels, and genders need to be developed for this population.

Table 10. Descriptive statistics of AFM_1 levels in Ghana from three separate time points

	AFM_1 (pg/mg creatinine)		
	June-Aug. 2004	Sep. 2005	October 2010
AFM_1 Positive	40.9%	86.7%	100%
Median	43	50	274
Mean ± S.D.	276 ± 883	361 ± 1,246	818 ± 1,235
Range	0 – 9,532	0 - 13,297	15-5,454

3. REDUTION IN THE URINARY AFLATOXIN M_1 BIOMARKER AS AN EARLY INDICATOR OF THE EFFICACY OF DIETARY INTERVENTIONS TO REDUCE EXPOSURE TO AFLATOXINS*

3.1 Introduction

In Sub-Saharan Africa, hepatocellular carcinoma (HCC) is one of the most common malignancies (Ferlay et al. 2010). In Ghana, HCC mortality accounts for 21.15 and 10.97% of all cancer related deaths in men and women, respectively (Wiredu and Armah 2006). Multiple factors play a role in the etiology of HCC; one major environmental risk factor is chronic exposure to AF from the diet. Inadequate food storage practices, little to no regulations on mycotoxin contamination, food insecurity and economic burdens make these populations at high risk for life-long exposure to harmful toxins

Toxin enterosorption strategies using calcium montmorillonite clay (NS) to reduce biomarkers of exposure and toxicity have been shown to be effective in animals and humans (Kubena et al. 1998; Ledoux et al. 1999; Phillips et al. 1990; Pimpukdee et al. 2004; Schell et al. 1993a). In the following study, UPSN was utilized to investigate the ability of AFM_1 biomarkers to rapidly indicate treatment efficacy.

Linear biomarkers, like AFM_1, are predictive of AFB_1 intake and are currently used as the standards for assessment of population exposures. Biomarkers are important tools in determining the efficacy of intervention trials for the reduction of AF exposure

* Reprinted with permission from "Reduction in the urinary aflatoxin M_1 biomarker as an early indicator of the efficacy of dietary interventions to reduce exposure to aflatoxins." By Mitchell NJ, Kumi J, Johnson NM, Dotse E, Marroquin-Cardona A, Wang JS, Jolly PE, Ankrah NA, and Phillips TD. *Biomarkers,* Aug; 18(5):391-398, Copyright 2013 by Informa UK Ltd.

in human populations due to the latent onset of AF related health consequences. The AFB_1-albumin marker indicates exposure over a number of weeks, whereas the AFM_1 marker is reflective of acute AF consumption allowing detection of AFM_1 24-48 hr after exposure. Previous work with a proposed AF binder in a human intervention trial showed significant decreases in both AFB_1-albumin and AFM_1 biomarkers following 3-months of treatment (Wang P et al. 2008). AFM_1 and the AF-mercapturic acid conjugate were decreased following 1 month of treatment with Oltipraz, a chemopreventative agent that affects phase 1 and phase 2 metabolism of AF (Wang JS et al. 1999). Although these biomarkers have proven useful to delineate efficacy of long-term interventions, shorter pilot trials are desirable, especially for use in children and other vulnerable groups, where appropriate dosimetry has not yet been defined. Based on the extant scientific literature, we postulate that daily AFM_1 levels could indicate efficacy over a short time period. Use of this biomarker as an indicator of efficacy would allow for smaller, non-invasive, pilot trials that could indicate probability of an intervention to reduce AF toxicity with minimal harm to human participants (especially the vulnerable). Following positive results from the AFM_1 biomarker, further human trials could be conducted to confirm safety, efficacy and health outcomes using long-term biomarkers.

Our objectives in this study were to: 1) determine the ability of AFM_1 biomarkers to act as an early indicator of treatment efficacy; 2) analyze the capacity of UPSN to significantly reduce AFM_1 levels in a short-term study in humans; and 3) assess the palatability of UPSN when included in Ghanaian foods.

3.2 Materials and methods

3.2.1 Materials

AFM_1 was obtained from Sigma Aldrich (St. Louis, MO, USA). Immunoaffinity columns were purchased from VICAM (Watertown, MA, USA). NovaSil (NS) clay was obtained from BASF/Engelhard Chemical Corporation (Iselin, NJ, USA). UPSN and calcium carbonate were purchased from Texas Enterosorbents, Inc. (Bastrop, TX). All materials designated for human consumption were treated with electron beam radiation prior to study initiation and were also analyzed for various metals and levels of chlorinated dibenzo-p-dioxin/furans by Columbia Analytical Services, Inc. (Houston, TX) (Marroquin-Cardona et al. 2011).

3.2.2 AFB_1 sorption analysis

Isothermal analyses of AFB_1 sorption onto surfaces of UPSN at equilibrium were performed according to methods reported by Grant and Phillips (1998) and described in detail by (Marroquin-Cardona et al. 2009). UPSN clay or calcium carbonate (50 ng) were mixed with 11 different concentrations of AFB_1, all done in triplicate, for 2 hr at a pH of 2 and 6.5. Samples were then centrifuged and the absorbance read at 362 nm using a Shimadzu scanning UV visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Computer-generated equilibrium isotherms were fit to the Langmuir model (based on r^2 values and randomness of the residuals). The parameters of Q_{max} and K_d were estimated to determine the maximum sorption to the surface and the affinity of the sorption interaction. Single point sorption analyses instead of isotherms were conducted

on the calcium carbonate as the data did not fit a Langmuir model and the percent reduction in AFB_1 was calculated for these values.

3.2.3 Study site and design

In recent years, studies with participants from the Ejura-Sekyedumase district of the Northern Ashanti Region of Ghana have proven that this community is at high risk for chronic AF exposure based on both AFB_1-albumin and AFM_1 levels (Jolly et al. 2006; Wang P et al. 2008). Study participants were recruited from five communities in this district. Sociodemographic data for these communities was established previously (Jolly et al. 2006). All recruited participants were between 21-70 years of age. Consent was sought following a community meeting with study personnel. Consent documents were translated and explained to participants in private rooms and signed by each individual participant before initiation of the study. Previous research from our laboratory in humans has shown that 3 g/day of NS clay was the minimal effective dose for significantly decreasing the AFM_1 biomarker (Wang P et al. 2008). This dose represents approximately 0.25% UPSN (w/w) of the total amount of food consumed daily (1200 g) by the average adult Ghanaian. Participants were selected evenly between the five communities (10 from each) and randomly assigned into one of two treatment groups. Figure 7 shows the overall study design and sample collection procedure. A local caterer prepared a breakfast and dinner meal for all participants daily. Participants were responsible for any snacks consumed and their lunch meals. Trained study monitors mixed each participant's treatment into their respective food before consumption. Each study participant received 1.5 g of placebo (calcium carbonate) or

UPSN in their breakfast meal and their dinner meal. Breakfast meals consisted of a corn-based porridge called "koko" and the dinner meals were a common soup (i.e. peanut soup, lamb lite soup) and corn dough called "banku". Treatment group 1 consumed placebo (3 g/day) in their foods for five days followed by a two day washout period and consumption of 3 g/day of UPSN for an additional five days. Treatment group 2 consumed UPSN (3 g/day) for the first five days followed by a 2 day washout period and an additional five days of placebo treatment (3 g/day). The crossover study design allowed for a smaller number of participants and each participant was used as its own control during data analysis to account for inter-individual variations in AF metabolism. Urine samples were collected at baseline, daily during treatments and at day 20. Overnight urine samples were collected daily, and 15 ml aliquots were stored at -20°C. Samples were transported cold to Noguchi Memorial Institute for Medical Research for biomarker analysis. Laboratory employees were blinded to treatment groups during analysis. Ethical clearance and institutional review board approval for this study was obtained from both Texas A&M University and Noguchi Memorial Institute for Medical Research (IRB 2009-0412 and 005/08-09).

3.2.4 AFM_1 analysis and palatability questionnaire

Urinary AFM_1 was analyzed through immunoaffinity column clean-up followed by HPLC coupled fluorescence detection (previously described in detail in Section 2.2.3). Following consumption of each meal participants were asked to rate the food based on four criteria; 1) overall taste, 2) texture, 3) aroma and 4) would they eat the food again. The first three criteria had the following rating options: poor, unacceptable,

acceptable or good while the fourth criterion was rated yes or no. Questionnaires were given to participants in English or translated to the local language by study monitors.

3.2.5 Statistical analysis

All statistical analysis was run on JMP 9 software (SAS Institute, Cary, NC, USA). AFM_1 data was not normally distributed; thus the data was analyzed with a nonparametric test (Kruskal-Wallis). However, all data was also analyzed under parametric conditions (ANOVA) following a log transformation of the data. Both parametric and nonparametric analyses were used to compare groups by days and by treatment arms. A p-value <0.05 (two-tailed) was considered significant. Statistical significance was not changed between parametric and nonparametric testing. Data was analyzed with participants acting as their own controls over two different time periods and with AFM_1 levels being compared between participants during a common time period. Data was also grouped by treatment for days 1-5 and grouped separately for days 8-12 and analyzed by ANOVA. Questionnaire data was analyzed categorically with a chi-square test by treatments.

3.3 Results

3.3.1 AFB_1 sorption analyses

The parameters of Q_{max} and K_d were derived for sorption of AFB_1 at both pH 2 and 6.5 onto UPSN. Isotherms are run at both a pH of 2 and 6.5 to simulate conditions the clay would encounter in the stomach and intestine. The sorption of AFB_1 onto surfaces of UPSN fit the Langmuir model ($r^2 \geq 0.92$) with an L-shape pattern indicating saturable binding at sites similar to those shown on parent NS clay (Figure 8A).

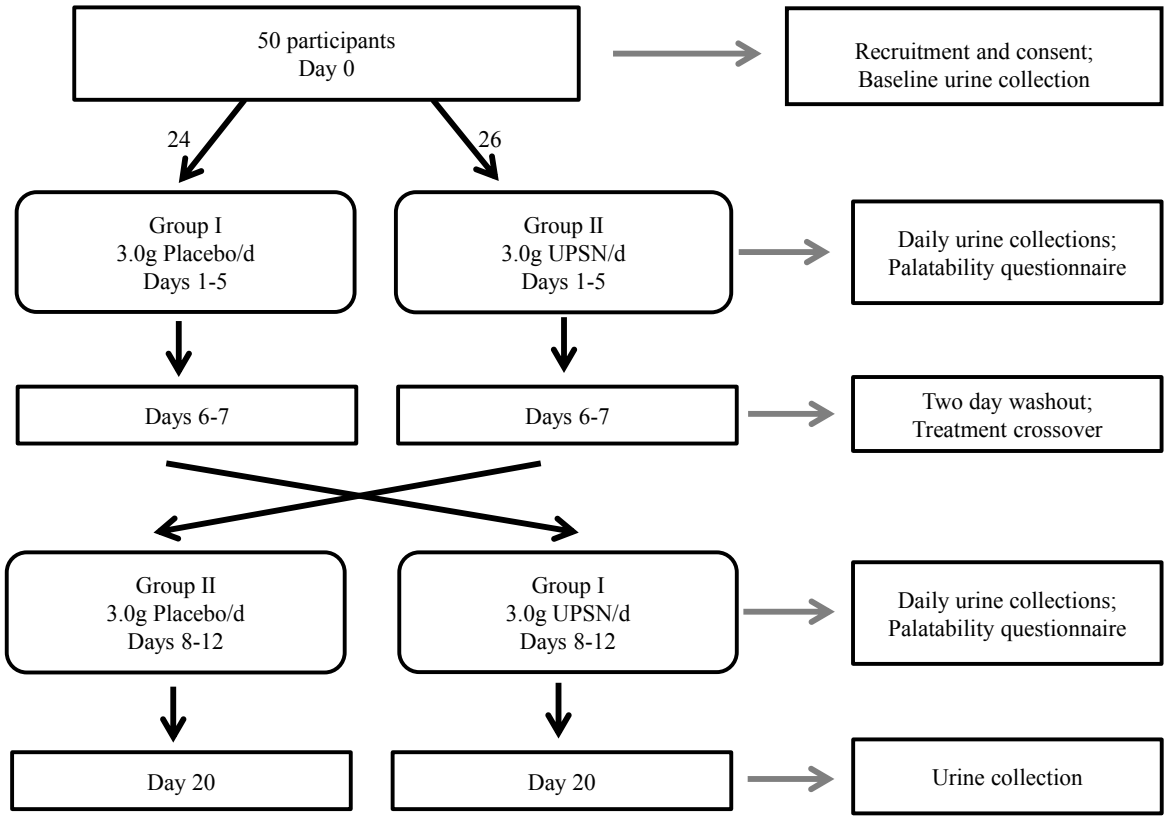


Figure 7. Crossover Study Design. Participants were randomly placed into one of two groups consuming 3.0 g of either calcium carbonate or UPSN per day. Following 5 days of treatment and 2 days of washout, groups were switched to the opposite treatment for another 5 days.

and 2 days of washout, groups were switched to the opposite treatment for another 5 days.

As previously reported by Marroquin-Cardona et al. (2011) the theoretical Q_{max} values calculated for UPSN were 0.39 ± 0.01 mol AFB_1 kg^{-1} and 0.44 ± 0.05 mol AFB_1 kg^{-1} at pH 2 and 6.5, respectively (Marroquin-Cardona et al. 2011). Although the Q_{max} valueare similar, Figure 8A clearly shows a separation in the two curves for UPSN based on pH. This difference can be further shown by the differences in the calculated affinity values for both curves ($K_d = 6.29x10^5$ at pH 6.5 and $1.93x10^5$ at pH 2). Figure 8A also shows the extrapolated isothermal curves for AFB_1 interaction onto the surfaces of calcium carbonate (placebo). The curves for calcium carbonate do not fit the Langmuir model and cannot be compared with the curves obtained for UPSN. As a result, single sorption assays were conducted in triplicate for calcium carbonate to obtain percent reduction of AFB_1. Maximum binding of AFB_1 with calcium carbonate occurred at 4 µg at both pH values with only 13% bound at pH 6.5 and 5% bound at pH 2 (Figure 8B). As the concentration of AFB_1 increases the percent bound decreases with pH 2 values dropping below zero. This data indicates a poor binding capacity of calcium carbonate for AFB_1 and the likely possibility that calcium carbonate is being dissolved under acidic conditions.

3.3.2 Sample collection and study population

A total of 50 participants were recruited for this intervention trial with 25 randomly placed into one of two treatment groups; 1) placebo days 1-5 and UPSN days 8-12, and 2) UPSN days 1-5 and placebo days 8-12. Groups had roughly the same number of males and females and similar age ranges (Table 11). The overall compliance among study participants and sample availability for biomarker analysis was satisfactory

with a total of 46 participants included in data analysis (Table 11). The four participants who were excluded from the study either missed two urine collections or treatment doses in a row.

3.3.3 Analysis of urinary AFM_1 levels

A total of 534 urine samples were collected and analyzed for AFM_1 over the course of the 20 day study. Mean, median and the range of AFM_1 for each group at different stages of treatment are shown in Table 12. All samples analyzed had detectable AFM_1, and no significant difference was found at baseline levels between the groups ($p=0.8737$). Comparisons to baseline were also assessed within each group during the various stages of treatment, with only day 20 values for group 2 being significantly lower ($p=0.0277$). There was a significant difference at day 20 between group 1 and group 2 ($p=0.0055$). Figure 9 shows daily mean urinary AFM_1 over the full 12 days of treatment and demonstrates the crossover in treatments for the groups with a switch in AFM_1 levels occurring by day 9. Comparisons between the UPSN treated groups and placebo groups were conducted for each time point by a one-way ANOVA of the log transformed data. Day 2 of the study was the only time point showing a significant difference between UPSN and placebo groups. Figure 10A shows the data grouped and analyzed using the participants as their own controls (i.e., average levels for each group from days 1-5 were run against average levels from days 8-12). There was no significant difference between placebo and UPSN treatment for group 1 ($p=0.1782$), however there was a significant difference between placebo and UPSN treatment for group 2 ($p<0.0001$) (Figure 10A). Differences in dietary intake, and thus AF intake during days

1-5 and days 8-12 resulted in large variability between treatment arms. Thus, the data was also analyzed by comparing groups during the same time period (i.e., group 1 vs. group 2 during days 1-5 and days 8-12) (Figure 10B). The average AFM_1 values were significantly decreased when placebo treatment was compared to the UPSN between groups during both days 1-5 (p=0.0011) and days 8-12 (p=0.0072) (Figure 10B).

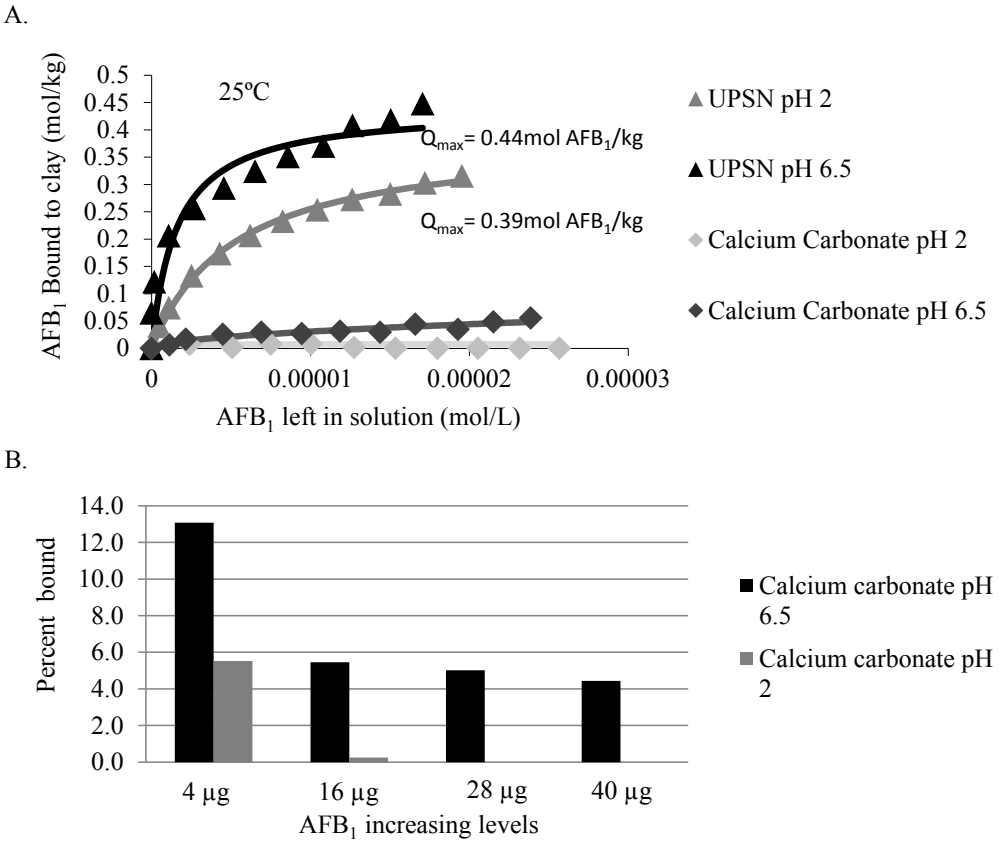


Figure 8. Isothermal AFB_1 sorption analysis of placebo and UPSN. A) Aflatoxin B_1 adsorption isotherms onto UPSN and calcium carbonate at pH 2 and 6.5. UPSN samples are depicted in an L-shape curve characteristic of a saturation of binding sites by AFB_1 molecules in a planar configuration (Marroquin-Cardona et al. 2011). Samples of calcium carbonate are also depicted; however, the curves did not fit the L-shaped pattern or the Langmuir model. B) Q_{max} values could not be derived for the calcium carbonate samples, thus percent bound values were calculated at single concentration points of AFB_1.

Figure 8. Isothermal AFB_1 sorption analysis of placebo and UPSN. A) Aflatoxin B_1 adsorption isotherms onto UPSN and calcium carbonate at pH 2 and 6.5. UPSN samples are depicted in an L-shape curve characteristic of a saturation of binding sites by AFB_1 molecules in a planar configuration (Marroquin-Cardona et al. 2011). Samples of calcium carbonate are also depicted; however, the curves did not fit the L-shaped pattern or the Langmuir model. B) Q_{max} values could not be derived for the calcium carbonate samples, thus percent bound values were calculated at single concentration points of AFB_1.

There was no significant difference between groups when both were on UPSN treatment (p=0.0804) or placebo treatment (p=0.2546). Median urinary AFM_1 had a 45% reduction with UPSN treatment during days 1-5 and a 55% reduction during days 8-12. There were no age or gender differences found with AFM_1 excretion between the study groups.

Table 11. Study Participant Demographics

	Females	Males	Age Range	Study Compliance
Group 1	9	11	25-70 yr	20/24
Group 2	11	15	21-46 yr	26/26
Total	20	26	21-70 yr	46/50

3.3.4 Palatability and adverse events

The percentage of foods that were deemed good or acceptable are listed in Table 13. Participants never deemed any food products as unacceptable or poor during the study, and all participants said that they would eat the food again. Both placebo and UPSN treated foods received a higher percentage of "good" ratings than "acceptable" ratings. Pearson chi-squared tests were used to analyze the difference between "good" and "acceptable" ratings within each group by placebo or UPSN treatment. There were no significant differences between placebo and UPSN when rating for taste, aroma or texture. During the 10 days of treatment there were two incidents of constipation reported that lasted 24 and 12 hr while one participant complained of diarrhea that lasted for 12 hr. However, these were isolated events that occurred while the respective

participants were taking placebo treatment and were not associated with UPSN consumption.

Table 12. Urinary AFM_1 excretion

Treatment stage	AFM_1 (pg/mg creatinine)			
	Baseline	Placebo	UPSN	Day 20
	288	389	281	480
Group1	831 ± 1279	1125 ± 2168	922 ± 1481	962 ± 1481
	(15-5455)	(4-17155)	(4-8018)	(14-6952)
	210	508	177	94
Group2	809 ± 1226	1287 ± 2550	483 ± 973	273 ± 1617 [a, b]
	(17-4925)	(4-20874)	(5-8397)	(10-1322)

Data are median, mean ± SD, and (data range). (a) Indicates a significant difference ($p<0.05$) between the same treatment stage when group 1 is compared with group 2. (b) Indicates a significant difference ($p<0.05$) from baseline within the same group.

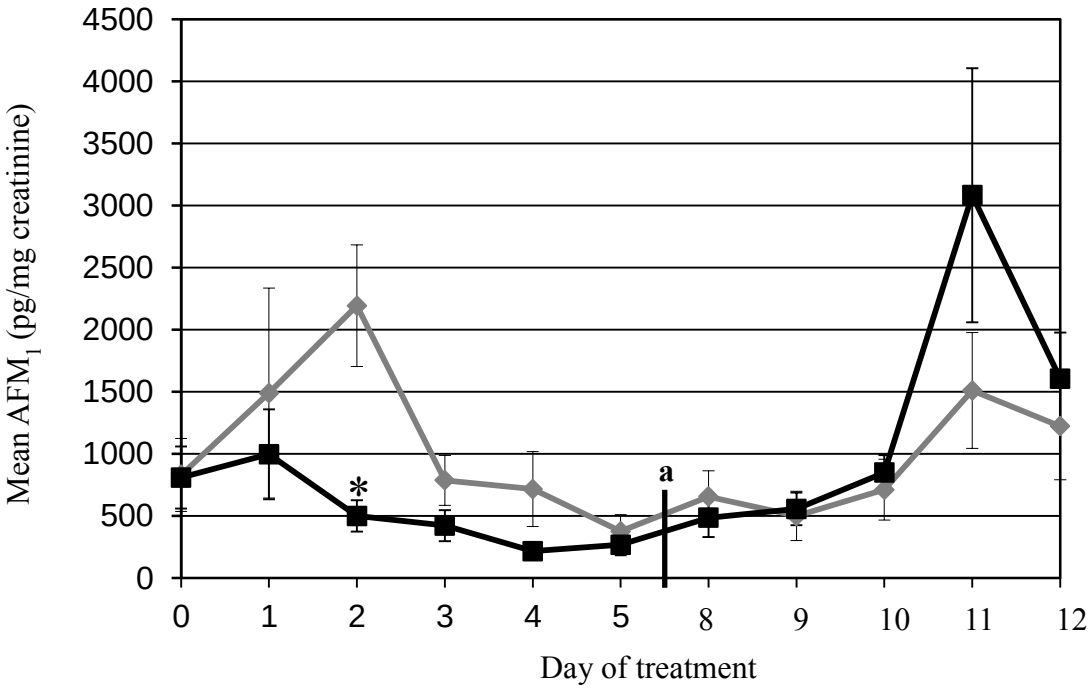


Figure 9. Daily mean AFM_1 levels. Points are mean values for each group per day and error bars represent standard error of the mean. Day zero represents baseline urine samples taken for participants. Group 1 (grey) was on placebo treatment days 1-5 and UPSN treatment days 8-12. Group 2 (black) was on UPSN treatment days 1-5 and placebo treatment days 8-12. * $p<0.05$ as compared with placebo at the same time point. (a) indicates the switch in treatment.

3.4 Discussion

AF-specific biomarkers currently consist of AFB_1 metabolites and AFB_1 adducts (Groopman et al. 1996; Groopman et al. 1994). Both serum the AFB_1-albumin adduct and urinary AFM_1 have shown significant correlation with dietary intake of AFs (Egal et al. 2005; Gan et al. 1988; Groopman et al. 1992b; Zhu et al. 1987). While the AFB_1-albumin adduct is considered to be the most stable and reliable biomarker for chronic AF exposure (Wild and Turner 2002), the long half-life of the biomarker requires exposures of one month to reach a steady state for analysis, which precludes its use in short-term human trials. AFM_1 in urine (Zhu et al. 1987) reflects recent AFB_1 exposure in humans,

and can be easily collected from both adults and children. Importantly, the short half-life makes it an excellent candidate for use in preliminary and pilot intervention trials.

Research involving human subjects, particularly children, should be conducted in a manner to reduce any possibility of harm. Utilization of the AFM_1 biomarker to prove efficacy could significantly decrease the time participants need to be treated with an investigational therapy and also lower overall invasiveness of such intervention trials. This study is the first to show that daily AFM_1 concentrations can be utilized to determine the efficacy of an intervention trial within 5 days of treatment implementation. The low cost, low invasiveness of sampling and short half-life of the AFM_1 biomarker, make daily sampling possible. This method could facilitate rapid surveillance of aflatoxicosis outbreaks and rapid identification of effective strategies to mitigate this disease.

This work not only shows the sensitivity of AFM_1 as a biomarker of intervention efficacy but also clearly illustrates the ability of a calcium montmorillonite clay, UPSN, to significantly decrease AF exposure within 5 days of treatment initiation. The immediate response observed in this study indicates that UPSN would be a prime candidate to treat populations during incidences of acute AF outbreaks. Clay as a toxincant enterosorbent can be delivered in various dose forms. In previous studies, NS clay was sterilized, encapsulated, and taken with water before each meal (Afriyie-Gyawu et al. 2008b).

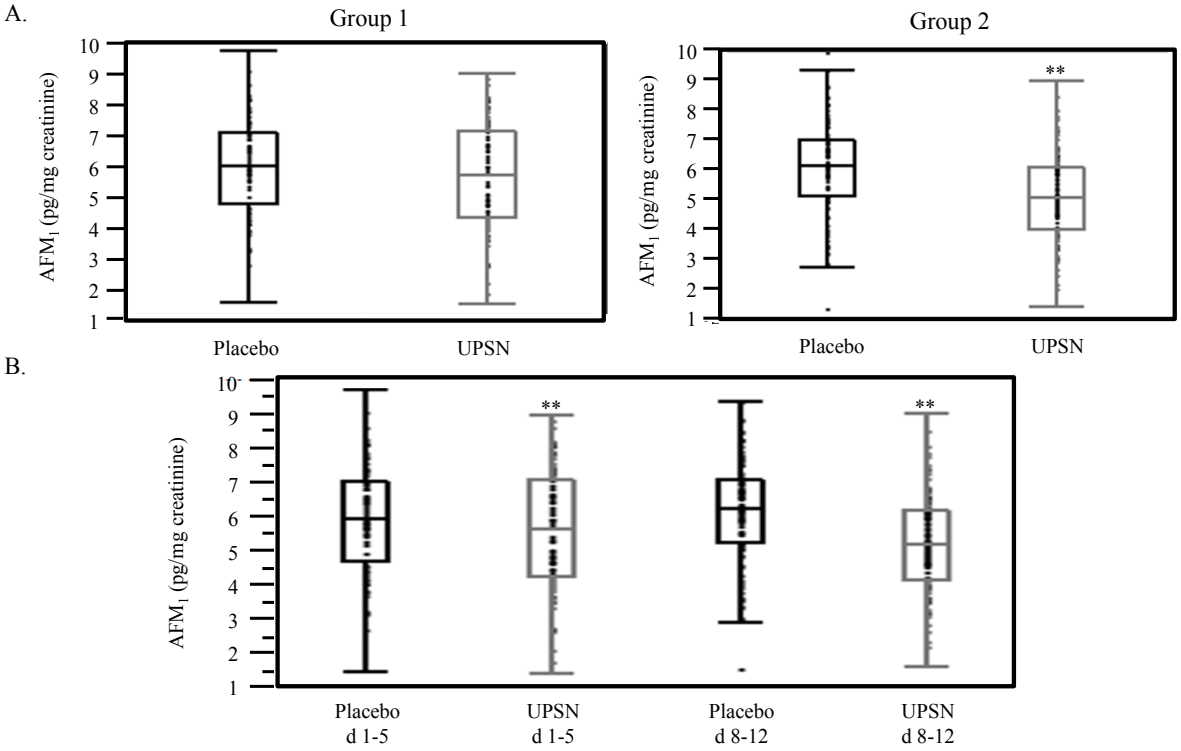


Figure 10. AFM_1 distribution within groups and treatment arms. The box values ranged from 25 to 75 percentiles of the total samples, the line within it indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively. A) Comparison of UPSN and placebo treatment within the same group. Analysis of the data in this manner allowed for each person to be used as their own control and account for inter-individual differences in AFB_1 metabolism and AFM_1 excretion. B) Distribution of AFM_1 levels by time points. Comparison of median AFM_1 levels were compared between groups based on time points to account for differences in daily dietary AFB_1 intake. Placebo days 1-5 was compared with UPSN days 1-5 and the same was calculated for days 8-12. **p<0.05 as compared with placebo.

Figure 10. AFM_1 distribution within groups and treatment arms. The box values ranged from 25 to 75 percentiles of the total samples, the line within it indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively. A) Comparison of UPSN and placebo treatment within the same group. Analysis of the data in this manner allowed for each person to be used as their own control and account for inter-individual differences in AFB_1 metabolism and AFM_1 excretion. B) Distribution of AFM_1 levels by time points. Comparison of median AFM_1 levels were compared between groups based on time points to account for differences in daily dietary AFB_1 intake. Placebo days 1-5 was compared with UPSN days 1-5 and the same was calculated for days 8-12. **p<0.05 as compared with placebo.

The cost and inconvenience of encapsulation led us to consider alternate dose forms. Utilization of the clay as a food additive could have numerous advantages including lowered cost of production, decreased impact on people's daily life, possible increased efficacy and improved sustainability. UPSN was developed as a more palatable form of the parent clay for inclusion in food products. UPSN contained a lower percentage of particles above 100 μm in the dry state and above 50 μm in the wet state versus parent NS clay (Marroquin-Cardona et al. 2011). Although X-ray diffraction (XRD) analysis identified quartz in the UPSN product, the relative intensity was lower than that of the parent clay suggesting less content of this mineral (Marroquin-Cardona et al. 2011). UPSN and NS had similar binding affinities for AFB_1 *in vitro* and were shown to contain the same active product (calcium montmorillonite) based on XRD intensities (Marroquin-Cardona et al. 2011). Based on the characterization of both montmorillonite clays, UPSN was deemed preferable for food inclusion in the present study.

This is the first *in vivo* study to assess the efficacy of UPSN and the first human intervention study to evaluate a form of NS clay as a food additive. Only one of the two groups (group 2) had a significant reduction in AFM_1 levels when comparing UPSN dosing with their placebo dose. The lack of significance in AFM_1 reduction for group 1 could be due to variations in dietary AFB_1 intake from days 1-5 when compared to days 8-12. The median exposure in placebo groups is 1.3 times higher during days 8-12 than days 1-5 making it hard to make meaningful comparisons between UPSN and the placebo treatments within the same group. Due to differences in dietary AF intake from

Table 13. Palatability ratings by percent

	Rating	Taste	Aroma	Texture
Placebo	Good	84.8	78.3	80.4
	Acceptable	15.2	21.7	19.6
	Unacceptable	0	0	0
	Poor	0	0	0
UPSN	Good	78.3	65.2	69.6
	Acceptable	21.7	34.8	30.4
	Unacceptable	0	0	0
	Poor	0	0	0

the first half and the second half of the study, the data was also compared between groups over the same time points. Inter-group comparisons showed a significant reduction in urinary AFM_1 levels in this study. A reduction rate of up to 55% in the median AFM_1 levels could be observed in as little as 5 days of treatment. These reduction rates are similar to those seen in other intervention trials utilizing other dietary supplements (i.e. NS clay, Chlorophyllin and oltipraz) based on acute biomarkers (AFM_1 and AFB-N^7-guanine) of exposure from urine (Egner et al. 2001; Wang JS et al. 1999; Wang P et al. 2008). In earlier research with NS, it took 3-months of treatment to see a

significant difference in urinary biomarkers (Wang P et al. 2008). However, in that study, urine samples were only taken twice throughout the 3-month period, and the large variability in biomarker output due to inter-individual differences of AF metabolism and dietary intake can lead to a lowered power of the analysis. In this study, daily sampling of the biomarker and pooling of the data decreased the effects of the variability of individual biomarker concentrations on the statistical analysis. A similar reduction in AFM_1 metabolites over a 3-month treatment period has been associated with a significant decrease in AFB_1-albumin adducts in humans (Wang P et al. 2008).

Recent reports of growth stunting associated with AF consumption (Gong YY et al. 2002, 2004 Turner et al. 2007) and AF contaminants occurring in children's ready to use therapeutic foods has caused significant concerns for child health and acute health impacts from high level exposures to dietary AFs in the vulnerable and susceptible. Populations that lie within what has been called the "hot zone", 40°N and S latitude, are the most commonly exposed to AF due to a favorable climate for *Aspergillus* growth and AF production (Cotty and Jaime-Garcia 2007b; Williams et al. 2004). A variety of strategies for reducing AF contamination in food and feed have been reported including the use of benign competitive fungal species, establishing drought resistant crops, food sorting and improved storage processes. However, none of these methods are suitable for therapy to alleviate acute aflatoxicosis and reduce lethality in the vulnerable in poor communities. Toxin enterosorbent intervention with UPSN clay has the capability to immediately impact exposure and rescue individuals suffering from acute and sub-acute AF exposures similar to those reported in Kenya in 2004.

4. PHASE I SAFETY INTERVENTION TRIAL OF UPSN TREATMENT IN THE DIETS OF CHILDREN IN GHANA

4.1 Introduction

Stunting, wasting, and fetal growth retardation result in more than 2 million deaths in children under the age of 5 and account for 21% of disability-adjusted life years worldwide (Black et al. 2008). In 2010, it has been estimated that 171 million pre-school children worldwide were stunted (de Onis et al., 2011). While the overall prevalence of stunting in developing countries has improved over the past two decades (from 44.4% to 29.2%), the majority of this improvement has been occurring in Asia and Latin America. The rate in Africa (40%), however, has remained mostly stagnant and is not expected to improve drastically over the next 10 years (de Onis et al. 2012). Although stunting is primarily attributed to nutritional and protein deficiencies, AF has also been associated with growth faltering in Sub-Saharan Africa (Gong YY et al. 2002, 2003, 2004; Okoth and Ohingo 2004; Shuaib et al. 2010; Turner et al. 2003, 2007; Wang E et al. 1992). Current strategies implemented to alleviate growth faltering and subsequent physical and mental deficits include food and micronutrient supplementation. These complementary foods, sometimes referred to as ready-to-use therapeutic foods (RUTFs), are designed to supplement the typical diet and often consist primarily of maize, groundnuts (peanuts), and other protein sources, such as soybeans (Bhutta et al. 2008; Hendricks KM 2010; Lartey et al. 1999). Recently, there have been reports of AF contamination in similar homemade complimentary foods (Weanimix) in Ghana, West Africa (Kumi et al. 2011).

Due to dietary exposures in African countries such as Guinea, Kenya, Benin, Togo, Senegal, and the Gambia, approximately 85% to 100% of children have detectable levels of serum or urinary AF biomarkers (Gong et al., 2003, 2004; Polychronaki et al., 2008; Turner et al., 2000, 2003, 2005; Wild et al., 1990, 1993). Therefore, it is clear that intervention strategies directed at the mitigation of child exposures are needed in areas where risk of AF consumption is high and malnutrition is common. Based on detailed studies conducted in animals and humans, it was determined that ingestion of UPSN at levels efficacious for reducing AFB_1 biomarkers would be reasonably safe in children.

In the following clinical trial, safety and efficacy of UPSN was assessed for children at risk for AF exposure from the Ejura-Sykedumase district of Ghana. The study followed a Phase I, double blind, placebo-controlled trial over a two-week time period. The results from this research will be utilized to design future studies investigating long-term protection of children at high risk for AF exposure and the potential of this material for short-term therapy during outbreaks of acute aflatoxicosis.

4.2 Materials and methods

4.2.1 Materials

UPSN was manufactured through a refinement process of the parent calcium montmorillonite, NS, by Texas Enterosorbents. UPSN was examined for various environmental contaminants, including dioxins and heavy metals to ensure compliance with federal and international standards. Metal and dioxin analysis of both NS and UPSN was reported to be similar and well under the tolerable daily intake (TDI) or provisional tolerable daily intake (PTDI) set forth by various food safety councils of

international organizations such as the World Health Organization (WHO) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Marroquin-Cardona et al. 2011). UPSN was sterilized by electron beam irradiation to ensure safety from any possible bacterial or viral contamination prior to trial initiation.

HPLC grade methanol, phosphate buffered saline, and AFM_1 standard were purchased from Sigma Aldrich (Saint Louis, MO, USA). Ultrapure deionized water (18.2 MΩ) was generated within the lab using an Elga™ automated filtration system (Woodridge, IL, USA). Immunoaffinity columns were purchased from VICAM (Watertown, MA, USA).

4.2.2 Study site and participant recruitment

Study participants were recruited from six communities in the Ejura-Sekyedumase district of the Ashanti Region of Ghana. The six communities included Dromonkuma, Hiawoanwu, Kotokoliline, Nkwanta, Ejurafie, and Kasei. These communities are in rural areas where inhabitants are primarily subsistence farmers. All recruited participants were between 3-9 years of age. Consent was sought from the parents or legal guardians following a community meeting with study personnel. Consent documents were translated and explained to each participant, then signed by each participant's guardian prior to initiation of the study. Participants were randomly assigned to one of three treatment groups. Figure 11 represents the overall study design and treatment arms. The three treatment arms consisted of a placebo group, which received 0.75 g calcium carbonate twice daily, a low-dose group which received 0.375 g UPSN twice daily, and a high-dose group which received 0.75 g UPSN twice daily. A

placebo-controlled group was deemed necessary in this research since clinical reference ranges for hematology and serum biochemistry values are not currently well established for African children (Zeh et al. 2012). Thus, the placebo group was used as a reference when determining safety of UPSN. Doses were weighed into identical packages at Noguchi Memorial Institute for Medical Research (NMIMR) prior to the study to ensure that monitors and participants would be blinded to their treatment. Trained study monitors mixed each participant's treatment into their breakfast and dinner meals before consumption. Breakfast meals consisted of a corn-based porridge called "koko" or soup and the dinner meals typically consisted of a common soup (i.e. peanut soup, lamb lite soup) and corn or cassava dough called "banku" and "fufu", respectively. These meals were supplied by the individual households. Participants provided blood samples (3 cc) on the morning prior to initiation of treatment (Day 0) and on day 15 (the morning after their last treatment dose). Blood samples were collected by trained phlebotomists at the Ejura District Hospital. Aliquots of the blood samples were used for hematological analysis and the remaining amount was centrifuged. The resulting serum was collected and kept at -20°C. Urine samples were collected by parents the morning of Day 0 (baseline), halfway through the study (day 7), and the morning after the final dose (day 15). Following collection, urine samples were stored at -20°C and together with the serum samples were transported to NMIMR for analysis. The study design followed the guidelines for a double-blind randomized Phase I clinical trial. Ethical clearance and Institutional Review Board approval for this study were obtained from both Texas A&M University and NMIMR in Accra, Ghana (2011-0684 and 043/11-12, respectively).

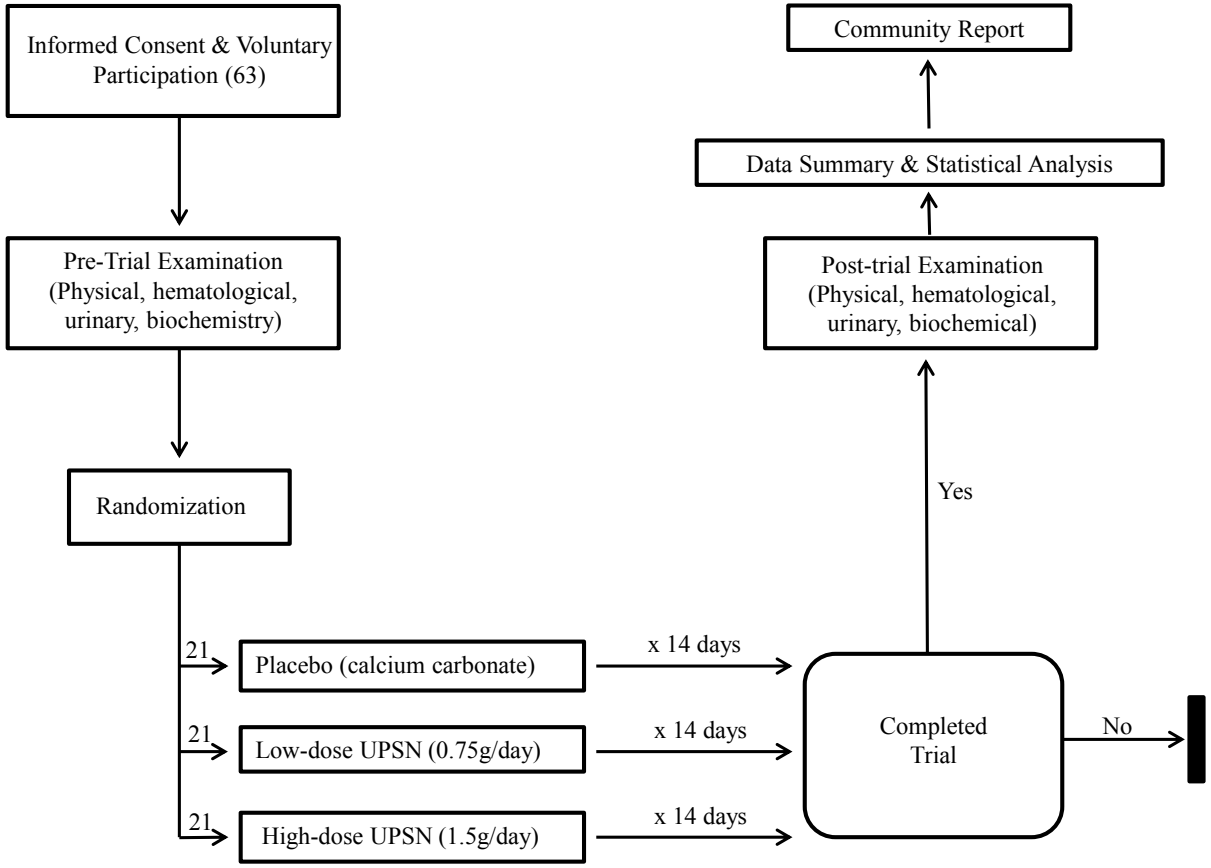


Figure 11. Phase I safety trial design. Flow chart delineating the study design and procedures for a 2-week clinical trial with UPSN in Ghana.

Figure 11. Phase I safety trial design. Flow chart delineating the study design and procedures for a 2-week clinical trial with UPSN in Ghana.

4.2.3 Toxicity and adverse events monitoring

Based on the existing scientific literature describing consumption of dioctahedral smectite clays in adults and children, no severe toxicity was expected as a result of UPSN treatment. However, research staff and medical personnel were on-site throughout the study period to monitor for potential adverse effects and to remove participants from the study in the event of such an effect. Daily diary worksheets and symptom checklists were provided to study monitors as assessment tools for adverse events monitoring and were completed twice daily following ingestion of each treatment dose. Adverse events are described as percentages of total number of adverse event reports out of the total number of completed daily diary worksheets per treatment group. In the event of an adverse treatment effect or unrelated condition at any time during the study, medical treatment was available to participants from the district hospital at no cost to the participant. Any symptoms were assessed according to the following criteria: Mild (grade 1), slightly bothersome and relieved with symptomatic treatment; Moderate (grade 2), bothersome and interfered with activities and only partially relieved with symptomatic treatment; Severe (grade 3), prevented regular activities and not relieved with symptomatic treatment. Any participant experiencing severe incidences were advised to seek immediate medical attention. Physical examination and laboratory analysis were performed for persistent symptoms. Any symptoms that were linked to the UPSN treatment by the study physician would result in immediate discontinuation of the treatment; however this did not occur during the study.

4.2.4 AFM_1 analysis, hematology, and serum biochemistry

Urinary AFM_1 was analyzed through immunoaffinity column clean-up followed by HPLC coupled fluorescence detection (previously described in detail in Section 2.2.3). Whole blood measurements consisted of hemoglobin, total white cell count and platelet count. Whole blood analysis was conducted with a flow cytometer (Abx micro60, Block Scientific, Bohemia, NY, USA). Serum albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total protein, total bilirubin, urea, creatinine, triglycerides, sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca^{2+}), and magnesium (Mg^{2+}) were measured using a Flexor E, automatic blood analyzer (Vital Scientific, Netherlands).

4.2.5 Statistical analysis

Statistical analysis was conducted with JMP 10 software (SAS Institute, NC, USA). The ultimate goal of this study was to determine if the ingestion of UPSN clay was safe in children; therefore, statistical evaluation focused on comparisons between treatment arms, as well as values within a group at baseline and at day 15. A Chi-square test was used for analysis of side-effect/toxicity data between treatment groups. As expected, AFM_1 biomarkers of exposure data was not normally distributed and was log transformed prior to analysis. Paired t-test and ANOVA statistical tests were conducted on both AFM_1 data and biochemical parameters for comparisons among treatment groups. A two-tailed p-value<0.05 was considered statistically significant. Correlation analyses were performed for serum biochemical parameters and AFM_1 levels. R-squared

values were based on a standard linear regression model; p-values and correlation coefficients were calculated by a Pearson correlation test.

4.3 Results

4.3.1 Study participant characteristics and compliance

In total, 63 children were enrolled in the clinical trial. There were no significant differences in mean age, gender, weight, or other general physical parameters such as blood pressure between treatment groups (Table 14). Adherence to the two-week study protocol was excellent, with all 63 participants completing the study. Only one participant missed an evening dose of treatment throughout the 28 doses/participant administered. This participant was in the low-dose UPSN group and was diagnosed with and treated for malaria that same day. General acceptance by the parents and children was exceptional.

4.3.2 Adverse events and side effects

The two dose levels of UPSN (0.75 and 1.5 g/day) were tolerable in the participants throughout the study. Adverse symptoms reported during the two-week study were primarily of a gastrointestinal nature and included vomiting and diarrhea. Table 15 is a tally of individual adverse events reported throughout the course of the study. It is important to note that, in some cases, reports were made multiple times by the same participant. For example, all reported events in the high-dose group originated from one individual who received medical attention from the district hospital following 2 consecutive days of vomiting. This participant was diagnosed and treated for malaria by doctors at the district hospital, but was allowed to stay in the clinical trial per the

physician's recommendation. Vomiting ceased after initiation of malarial medication. In total, there were 3 children diagnosed and treated for malaria during the study. This is equivalent to 4.8% of our study population, which is actually a lower prevalence rate than reported for the area (> 75 per 1,000) by the WHO (World Malaria Report 2012). The symptoms reported by individuals with malaria accounted for 9 out of the total 13 adverse events (69.2%) reported. The placebo, low-dose, and high-dose groups experienced side effects at a rate of 0.3% (2/588), 0.8% (5/588), and 1% (6/588), respectively. Severities of side effects were generally reported as mild to moderate and either no treatment or self-treatment was effective in alleviating symptoms. Severe cases of vomiting requiring immediate medical attention occurred only in those participants later diagnosed with malaria. Importantly, there were no significant differences observed in number of adverse events between treatment groups ($p>0.37$) or in severity of symptoms reported ($p>0.43$).

4.3.3 Hematological, blood chemistry, and serum mineral effects

Hematological analysis of blood samples showed no significant difference between treatment arms, although lymphocytes and monocytes were above the normal range across all treatment groups at both day 0 and day 15 (Table 16). Hematological values in the placebo group were not significantly different from either the low-dose or high-dose UPSN groups at day 0 or day 15. These levels were also not significantly different within each treatment group when comparing day 15 to baseline values.

Table 14. Demographics and Physical Parameter

	Treatment group		
Demographic Characteristics	Placebo	Low-dose	High-dose
Participants	21	21	21
Gender			
Male	13	14	14
Female	8	7	7
Age (years) [a]	5.8 ± 1.6	5.4 ± 1.8	5.7 ± 1.9
Body weight (kg) [a]	20.0 ± 4.8	21.0 ± 8.5	18.9 ±5.2
Height (cm) [a]	107.2 ± 13.2	107.9 ± 16.3	105.8 ± 15.1
Systolic blood pressure (SBP) (mmHg) [a]	87.7 ± 8.0	91.3 ± 10.9	92.3 ± 11.0
Diastolic blood pressure (DBP) (mmHg) [a]	44.6 ± 7.3	45.6 ± 9.1	47.2 ± 10.2

[a]Mean ± SD; Note: all data are baseline vales.

Results of serum biochemistry analyses are provided in Table 17. No significant differences were observed within groups between day 0 and day 15 for albumin, ALP, AST, GGT, total bilirubin, urea, or triglycerides. ALT values were significantly higher after treatment for all groups ($p < 0.0003$), including the placebo group. The placebo group also exhibited significantly lower ($p < 0.01$) total protein levels following treatment, however, this was not the case for the UPSN treated groups. At day 15, the low-dose UPSN group had significantly lower creatinine levels compared to baseline ($p<0.05$), however this observation was not dose-dependent. Comparison of serum biochemistry parameters between the placebo group and the low-dose or high-dose

UPSN groups did not indicate significant differences at day 0 or day 15 (Table 17). Although average total bilirubin levels were within range in all treatment groups throughout the study, a wide range of values were detected (0.2-35.5 μmol/L) and these values were positively correlated, with urinary AFM_1 concentration at day 0 (Figure 12A). Upon comparison of all treatment groups at day 15, the association was lost (Figure 12B). Interestingly, when AFM_1 decreased or increased from day 0 to day 15, there was a corresponding decrease or increase in bilirubin levels 62% of the time; indicating that bilirubin levels may have a positive association with AFM_1 excretion in children.

Median, mean, and normal U.S. pediatric ranges for the serum minerals assessed are outlined in Table 18. Serum Na^+ and Cl^- significantly increased ($p< 0.01$) over the course of the study in all groups. All groups exhibited significantly decreased Ca^+ levels following the study. A reduction in serum Mg^{2+} was seen in the UPSN high-dose group ($p< 0.01$). There was also a small reduction in the mean Mg^{2+} of the UPSN low-dose group, however it was not significantly different ($p>0.27$). However, levels in all groups remained well within the normal U.S. range. No other mineral level comparison between day 0 and day 15 was significantly different.

4.3.4 AFM_1 biomarker levels in urine

Throughout the study, 100% of urine samples contained detectable levels of AFM_1. AFM_1 concentration ranged from 0.5-5443.7 pg/mg creatinine. The mean at day 0 was 297.8 pg/mg creatinine. AFM_1 levels were not significantly different by gender or correlated with age (data not shown). Although treatment groups were not significantly

different at baseline or day 15 of treatment, the high-dose UPSN group exhibited significantly (p=0.216) lower AFM_1 levels on day 7 than both the placebo and low-dose group (Figure 13A-C). When all data values from day 7 and 15 were pooled by treatment group the high-dose group showed an overall statistically significant (p=0.0063) 70% reduction in AFM_1 levels when compared to control (Figure 14).

Table 15. Adverse Events Reported

	Treatment Group			
	Placebo	Low Dose	High Dose	Sum
Symptom Reported				
Other	0[a]	0	2 *	2
Indigestion	0	0	0	0
Nausea	0	0	0	0
Vomiting	2	3 *	4 *	9
Constipation	0	0	0	0
Diarrhea	0	1	0	1
Flatulence	0	0	0	0
Loss of Appetite	0	1	0	1
Abdominal Discomfort	0	0	0	0
Heartburn	0	0	0	0
Dizziness	0	0	0	0
Insomnia	0	0	0	0
Bloating	0	0	0	0
Total Incidence (%)	2 (0.3)	5 (0.8)	6 (1)	13 (0.7)
Severity				
Mild	2	2	5	9
Moderate	0	2	1	3
Severe	0	1	0	1

[a]Indicates number of times an adverse event was reported.
*Indicates participant was diagnosed with malaria by health official

Table 16. Hematological Analysis

	Treatment Group						Normal clinical range (US)	Reference
	Placebo		Low Dose		High Dose			
	Baseline	After	Baseline	After	Baseline	After		
Hemoglobin (g/dL)	11.3 ± 0.8	11.4 ±1.1	11.5 ± 0.9	11.7 ± 1.3	11.0 ± 1.5	11.3 ± 1.5	11.0-14.5	A
WBC	8.2 ± 2.0	7.4 ± 1.2	7.8 ± 2.0	7.9 ± 1.8	8.5 ± 3.4	8.2 ± 2.7	3.4-12.0	A
Platelet	275.3 ± 73.9	303.0 ± 112.4	296.0 ± 85.0	349.9 ± 142.7	266.0 ± 80.4	280.9 ± 117.3	150.0-450.0	A
Lymphocytes (%)	55.2 ± 10.9	56.9 ± 8.1	52.6 ± 7.9	51.8 ± 9.6	52.9 ± 11.2	53.5 ± 9.4	28.0-48.0	B
Monocytes (%)	10.6 ± 3.2	11.0 ± 3.9	12.3 ± 3.7	13.0 ± 3.9	10.4 ± 3.0	10.9 ± 3.3	3.0-6.0	B
Granulocytes (%)	34.2 ± 12.1	31.9 ± 7.4	35.1 ± 8.4	35.2 ± 10.7	36.6 ± 11.0	35.6 ± 9.1	32.0-76.0	B

Data are mean ± SD; A) Mayo Clinic pediatric reference values B) Children's hospitals and clinics of Minnesota reference values; Reference ranges are combined for males and females WBC and Platelet values are in (X 1000/mm^3).

Table 17. Serum biochemistry

	Treatment Groups						Normal Clinical Range (US)	Reference
	Placebo		Low Dose		High Dose			
	Baseline	After	Baseline	After	Baseline	After		
Albumin (g/L)	49 47.0 ± 9.0 15.9-56.0	47.2 46.9 ± 2.8 39.7-51.0	48.5 48.5 ± 3.1 42.5-53.0	47.9 47.2 ± 2.3 42.2-51.6	49.6 46.2 ± 11.1 11.1-57.4	48.4 47.6 ± 6.0 25.2-56.2	35.0-50.0	A
ALP (U/L)	591.9 559.1 ± 185.7 176.9-1039.4	596.2 532.0 ± 355.6 6.3-1164.3	479 427.2 ± 242.3 17.3-843.4	620.5 572.3 ± 332.6 6.1-1102.2	521.3 488.5 ± 215.8 13.1-907.6	581.9 584.9 ± 314.7 24.7-1189.1	149.0-468.0	A
ALT (U/L)	13.2 14.4 ± 6.0 4.3-27.1	27.1 30.2 ± 11.3 * 13.7-51.4	17.2 19.3 ± 8.6 7.5-46.5	27.6 28.9 ± 7.2 * 20.8-45.8	17.7 20.2 ± 20.0 3.0-87.3	24.6 29.3 ± 15.1 * 12.4-68.3	7.0-55.0	A
AST (U/L)	39.8 41.8 ± 13.2 16.8-66.3	41.2 44.9 ± 12.3 31.4-73.3	38.3 42.3 ± 8.4 30.9-59.5	42.1 43.7 ± 10.8 29.8-74.3	38.4 40.2 ± 14.1 12.6-85.9	40.4 41.4 ± 10.0 20.6-57.3	8.0-60.0	A
GGT (U/L)	15 16.6 ± 8.0 1.7-42.0	16.6 17.0 ± 6.8 6.1-31.4	10.6 13.9 ± 8.6 6.4-43.2	14 16.0 ± 7.3 7.4-35.5	13.2 14.1 ± 9.0 0.5-37.2	15 17.0 ± 11.7 2.8-48.7	7.0-29.0	A

Table 17. Continued

	Treatment Groups						Normal Clinical Range (US)	Reference
	Placebo		Low Dose		High Dose			
	Baseline	After	Baseline	After	Baseline	After		
	5.5	8.1	6.2	7.5	6.7	6.1		
Total Bili(µmol/L)	7.1 ± 7.1	8.5 ± 4.9	7.8 ± 5.4	7.9 ± 3.4	8.4 ± 8.1	8.3 ± 7.4	1.7-17.1	A
	0.2-28.1	0.6-22.4	0.5-25.4	1.8-15.9	1.1-35.5	0.2-29.9		
	80.5	76.3	77.8	77.2	79.8	80.9		
Total Protein (g/L)	78.8 ± 8.8	77.0 ± 3.3 *	78.2 ± 6.0	77.4 ± 4.3	79.8 ± 31.0	78.0 ± 8.6	63.0-79.0	A
	43.6-87.2	70.8-82.5	70.2-89.8	70.1-86.1	21.3-194.2	47.8-89.6		
	2.9	2.7	2.8	2.7	3.1	2.8		
Urea (mmol/L)	2.7 ± 0.8	2.9 ± 0.6	2.8 ± 0.8	2.6 ± 0.6	2.9 ± 1.2	2.9 ± 1.0	2.5-7.1	A
	0.8-3.8	2.1-4.5	1.5-4.6	1.6-3.7	0.6-5.2	1.6-6.0		
	58.1	53.3	61.7	54.3	64.4	59.1		
Creatinine(µmol/L)	54.6 ± 16.4	54.3 ± 6.2	62.4 ± 10.1	55.7 ± 9.5 *	60.7 ± 17.9	56.5 ± 9.0	50.0-110.0	C
	5.4-71.1	40.2-65.1	38.7-77.0	42.4-78.5	18.5-90.4	34.1-74.2		
	0.8	1.1	1	1	0.9	1.1		
Triglycerides (mmol/L)	1.0 ± 0.7	1.3 ± 0.8	1.3 ± 0.7	1.2 ± 0.7	1.6 ± 3.0	1.3 ± 0.6	<1.02	A
	0.2-2.6	0.4-3.5	0.5-3.0	0.7-3.8	0.2-14.1	0.5-3.2		

Data represent median, mean ± SD, and range; A) Mayo Clinic pediatric reference ranges C) Royal College of Physicians and Surgeons of Canada ranges; Reference ranges are combined male and female values

* Denotes significant difference between baseline and after treatment

4.4 Discussion

Chronic childhood AF exposure has gained interest over the past decade as a potential variable in the complex milieu of biological and environmental factors that lead to stunting, wasting, and suppressed immunity. In particular, Sub-Saharan Africa has been identified as an area at high risk for AF exposure as well as growth stunting. Investigations from the Ejura district in the Ashanti Region of Ghana have demonstrated on-going, high level AF exposure over the past decade (Jolly et al. 2006, 2011; Mitchell NJ et al. 2013; Wang P et al. 2008). A prevalence of up to 54.9% has been reported for stunted and/or wasted children from another district of the Ashanti Region (Inungu 1995). Although the high rates observed in this population are primarily thought to occur as a result of inadequate nutrition and protein intake, multiple variables likely contribute to the etiology of disease. Chronic AF exposure in this community could be one such contributing factor, particularly following administration of nutritional supplements such as homemade "weanimix", which consists of groundnuts, beans, and maize (0.5:0.5:4). A recent assessment of the weaning foods produced in this community, intended for children between the ages of six months and two years, showed AF contamination in 100% of samples with levels as high as 500 ppb (Kumi et al. 2011). Urine samples collected from children before and after 21 days of homemade weanimix consumption revealed increased levels of AFM_1 biomarkers, indicating that while it is an important nutritional supplement in this region, weanimix can also cause heightened AF exposure (Kumi et al. 2011). Therefore, an intervention strategy to reduce childhood exposure in

these populations, while maintaining the use of these important nutritional supplements, is of particular interest.

Enterosorption therapy may be a valuable tool in low-economic, high risk areas, where food insecurity results in a limited variety in the diet and continued consumption of poor quality foods (Phillips et al. 2008; Shephard 2003). Clinical trials utilizing similar dioctahedral smectite clays at doses as high as 6 g/day for the treatment of acute diarrhea in children resulted in limited adverse effects; of which mild constipation was the most severe event reported (Dupont et al. 2009; Lexomboon et al. 1994; Madkour et al. 1993; Szajewska et al. 2006). Similarly, results from the current study indicate that administration of dietary UPSN powder at concentrations from 0.75-1.5 g/day in healthy children (ages 3-9 years) for 14 days resulted in minimal side effects. Neither, dose-dependent toxic effects nor severe clinical symptoms were related to UPSN consumption in the present study. Hematologic parameters indicated that UPSN treatment did not impair immunity or promote an inflammatory response (George-Gay and Parker 2003).

ALT values were increased in all treatment groups at day 15; however there were not significant differences between the low-dose or high-dose UPSN groups, and placebo. These values were also within normal pediatric reference ranges reported by the United States Mayo Clinic (Table 17). Additionally, all other liver toxicity parameters (i.e. ALP, AST, bilirubin, and GGT) were not increased at day 15 in any treatment groups. Therefore, the cause of an increase in ALT values over the duration of the study remains unclear. Na^+ and Cl^- levels were also significantly increased over the study period, however this was observed in the placebo group as well as the UPSN treated

groups. This indicates that these changes were most likely due to a change in dietary consumption of Na^+ and Cl^-, possibly through higher intake of salt during the study period. The levels for Cl^- were still within normal reference range, while some Na^+ values were out of range based on the United States pediatric values as seen in Table 18 (Mayo 2013).

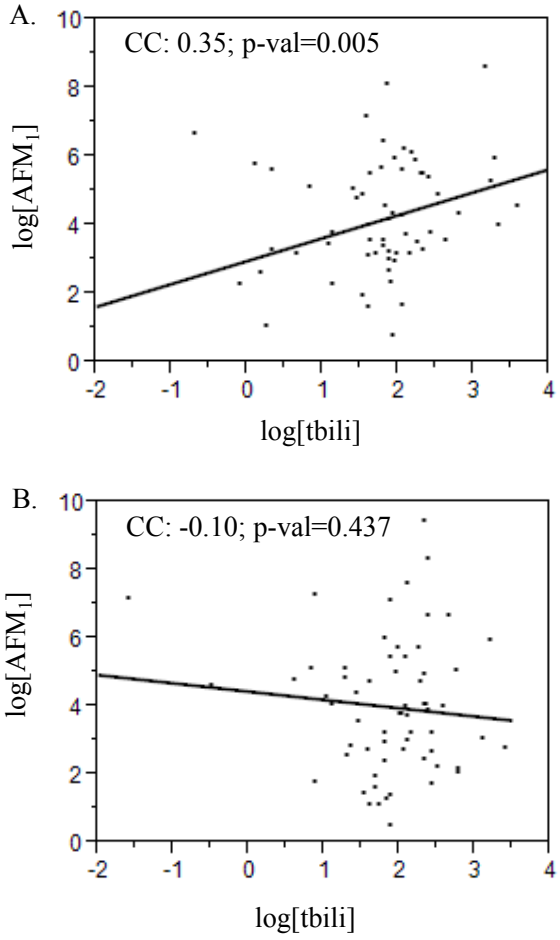


Figure 12. Correlation of log transformed total bilirubin and log transformed AFM_1 values for all participants at day 0 (A) and day 15 (B).

Figure 12. Correlation of log transformed total bilirubin and log transformed AFM_1 values for all participants at day 0 (A) and day 15 (B).

Increases in Ca^{2+} have been observed following administration of the parent NS product (Afriyie-Gyawu et al. 2005) and UPSN (Marroquin-Cardona et al., 2011) in rats, which was attributed to dissolution of calcite and exchangeable Ca^{2+} ions from montmorillonite. However, in the present study total Ca^{2+} levels were decreased in all treatment groups including the placebo (calcium carbonate). Calcium carbonate, and to a lesser extent NS clay, typically act as calcium supplements, thus it is likely that this overall reduction in serum levels is a result of dietary changes during the intervention trial.

Mg^{2+} was the only serum micronutrient that dose-dependently decreased with UPSN treatment. The mean concentrations in the high-dose group were significantly lower than the placebo group on day 15 following treatment and the low-dose group, while not significantly different, demonstrated a decreasing trend from the placebo. However, it is important to note that all levels remained within normal range throughout the study. Furthermore, significant modulation in serum Mg^{2+} concentrations have not been observed in any other animal or human study with UPSN or parent NS (Gelderblom et al. 1996a). Decreased absorption and retention of Mg^{2+} was observed in a pig model following ingestion of 1% of a sodium montmorillonite clay (Schell et al., 1993). Mg^{2+} plays an important role in many human metabolic functions, acting as a co-factor in enzymatic reactions that involve adenosine triphosphate (ATP). Levels are controlled by the kidneys and GI tract and appear to be closely linked to calcium, potassium, and sodium metabolism (Reinhart 1988). Therefore, the change in Mg^{2+} observed here could have resulted from changes in calcium or sodium metabolism and

not directly from UPSN treatment. An alternative explanation for the lowered serum Mg^{2+} observed is a direct sequestration of Mg^{2+} by UPSN in the gut through cation exchange activity of the clay, thus reducing the availability of Mg^{2+} for absorption from the gut. Longer safety trials controlling for intake of essential dietary nutrients are warranted to determine whether UPSN could interfere with micronutrient or mineral absorption in children.

Although changes in serum bilirubin have been reported following exposure to AF in animal species (Clifford and Rees, 1967; Clark et al., 1987) to our knowledge there have been no correlations made between AF exposure and bilirubin levels in humans. AF-alb, while a valuable AF assessment tool in the serum, is a long-term biomarker of exposure and is not known to fluctuate with recent exposure as rapidly as the urinary biomarker. For this reason, urinary AFM_1 is a better marker to correlate with rapidly changing serum components, such as bilirubin, which can modulate considerably in one day. As stated previously, AF has been shown to elevate total bilirubin and ALP levels in animal models. Since growth stunting has been reported to be common in most forms of chronic liver disease (Sokol and Stall, 1990), it will be important to assess liver function parameters in future studies involving children, growth-stunting, and AFs. Additionally, since direct bilirubin can be measured in the urine of individuals experiencing liver malfunction, this may be an excellent non-invasive biomarker to monitor in clinical AF studies.

Although the range of AFM_1 excretion was similar the average levels were significantly lower in this study than those seen in adults from the same population in

Table 18. Serum minerals

	Treatment Groups						Normal Clinical Range (US)	Reference
	Placebo		Low Dose		High Dose			
	Baseline	After	Baseline	After	Baseline	After		
Na (mmol/L)	132.2 130.6 ± 6.8 115.3-141.4	138.2 139.2 ± 5.5 * 132.2-153.4	135.3 133.6 ± 8.0 114.3-146.2	138.1 138.8 ± 3.8 * 134.5-150.3	128.5 129.3 ± 9.1 112.1-149.9	135.6 136.8 ± 3.1 * 132.7-143.3	135.0-145.0	A
K (mmol/L)	3.7 3.8 ± 0.4 3.0-4.8	4 4.0 ± 0.4 3.3-5.0	3.8 3.8 ± 0.4 2.7-4.5	3.9 4.1 ± 0.5 3.2-5.7	3.7 3.8 ± 0.5 3.1-4.8	4.1 4.1 ± 0.4 3.3-4.7	3.6-5.2	A
Cl (mmol/L)	97.3 97.4 ± 4.2 89.4-105.3	101.2 101.2 ± 4.0 * 90.1-108.4	98.8 97.7 ± 5.6 83.0-106.8	102.4 101.5 ± 2.5 * 96.0-104.8	96.2 96.6 ± 5.1 87.8-106.3	100.4 100.3 ± 2.1 * 95.4-105.2	102.0-112.0	A
Ca (mmol/L)	2.4 2.4 ± 0.2 1.9-2.7	2.1 2.2 ± 0.3 * 1.8-2.8	2.3 2.3 ± 0.1 2.0-2.6	2.2 2.2 ± 0.2 * 1.2-2.6	2.3 2.3 ± 0.2 2.0-2.6	2.1 2.1 ± 0.3 * 1.5-2.5	2.4-2.7	A
Mg (mmol/L)	0.9 0.8 ± 0.1 0.5-1.0	0.8 0.8 ±0.1 0.6-1.0	0.9 0.9 ± 0.1 0.7-1.0	0.8 0.8 ± 0.1 0.6-1.0	0.8 0.8 ± 0.1 0.7-1.1	0.8 0.8 ± 0.1 * 0.6-0.9	0.7-1.0	A

Data represent median, mean ± SD, and range; A) Mayo Clinic pediatric reference ranges; Reference ranges are combined male and female values.

* Denotes statistical significance between baseline and after treatment.

October of 2010 (Mitchell NJ et al. 2013). This finding may be explained by the fact that this intervention trial was carried out during the wet season, whereas the adult study took place at the beginning of the dry season, which typically correlates with increasing AF exposure (Turner et al. 2000; Wild et al. 2000). Also, the variance in excretion levels could be attributed to the difference in food intake, metabolism, and urinary output between children and adults. The high-dose UPSN group showed a significant decrease in AFM_1 excretion when compared to baseline levels for that group and when compared to controls (70%). This decrease in AFM_1 is higher than the percentages previously reported following NS and UPSN consumption in adults (58.7 and 55.0%, respectively) (Mitchell NJ et al. 2013; Wang P et al. 2008). The variation could be attributed to different inclusion levels of clay from the adult and children studies. Percent reduction in the adult clinical trials was based on an intake of 3 g clay/day while intake for the current study was 1.5 g/day. Although the amount of UPSN consumed in this clinical trial was half that of the adult study, it could account for a higher percentage of the total daily food intake, and thus the total daily AF intake. It is difficult to determine the actual daily caloric intake for this population, but this should be considered in future work when determining appropriate dose levels for vulnerable populations.

The results from this Phase I clinical trial indicate that UPSN consumption by children (ages three to nine years) is safe at a dose up to 1.5 g/day for two weeks. Inclusion of UPSN in weaning foods could also significantly decrease the amount of AFB_1 absorbed through the gastrointestinal wall, thereby reducing adverse effects of AF exposure and enhancing the quality, efficiency and safety of nutritional supplements.

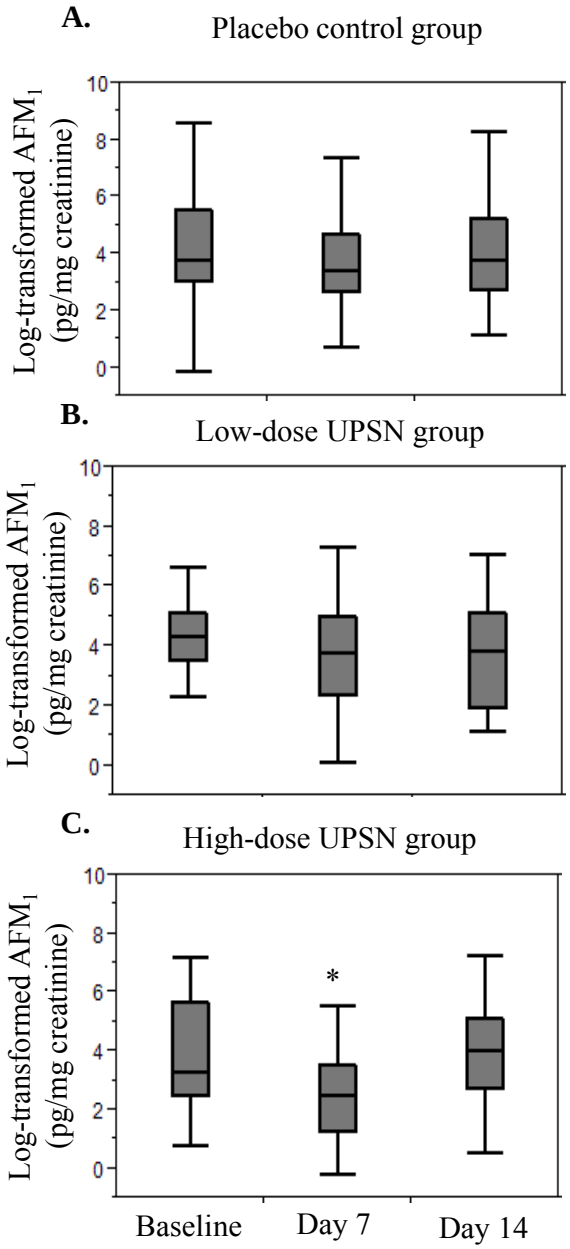


Figure 13. Dose effects of UPSN intervention of urinary AFM_1 excretion over the duration of the study. The box plots show distributions of AFM_1 levels in each group across time points. The box values range from 25 to 75 percentiles of the total samples, the line within the box indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and 75 to 95 percentiles, respectively. * Indicates statistical significance ($p<0.05$).

Figure 13. Dose effects of UPSN intervention of urinary AFM_1 excretion over the duration of the study. The box plots show distributions of AFM_1 levels in each group across time points. The box values range from 25 to 75 percentiles of the total samples, the line within the box indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and 75 to 95 percentiles, respectively. * Indicates statistical significance ($p<0.05$).

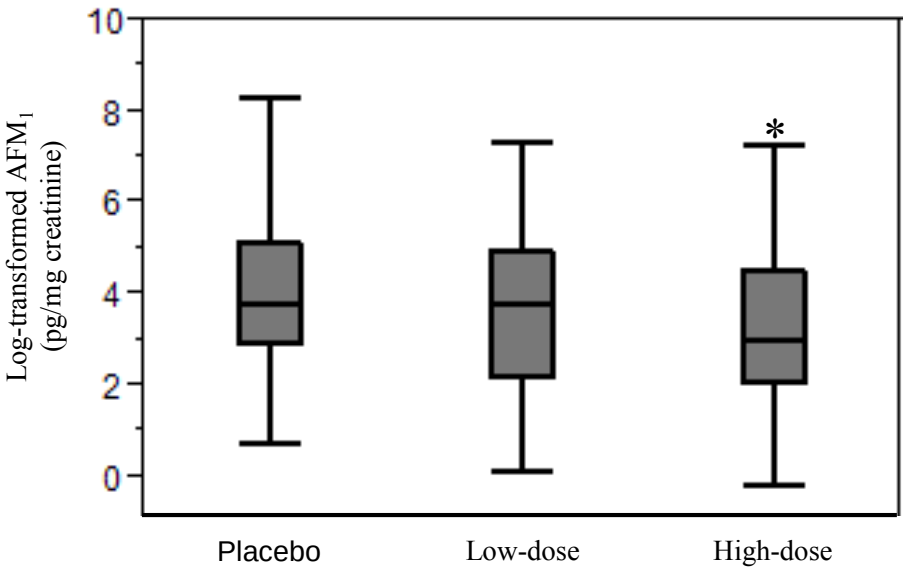


Figure 14: Comparisons of treatment groups following pooling of the AFM1 data for day 7 and day 15. The box values range from 25 to 75 percentiles of the total samples; the line within the box indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and 75 to 95 percentiles, respectively. *Indicates statistical significance ($p<0.05$).

Figure 14: Comparisons of treatment groups following pooling of the AFM1 data for day 7 and day 15. The box values range from 25 to 75 percentiles of the total samples; the line within the box indicates the median value. The bars on both sides of a box represent values ranging from 5 to 25 and 75 to 95 percentiles, respectively. *Indicates statistical significance ($p<0.05$).

5. TOXICOKINETICS OF AFB_1 AND FB_1 COMBINED EXPOSURE AND SORPTION WITH UPSN CLAY IN A RODENT MODEL

5.1 Introduction

Naturally occurring foodborne carcinogens, such as AFB_1 and FB_1, are predominantly investigated as independent compounds. However, complex mixtures of mycotoxins occur in the environment and could induce additive, antagonistic or synergistic interactions when present together (Carpenter et al. 1998). Research has demonstrated that multiple mycotoxins can co-contaminate crops and foods intended for both animal and human consumption (Almeida et al. 2012; Kimanya et al. 2008; Sun G et al. 2011). Although research investigating the health effects due to co-exposure of these two mycotoxins is limited, several studies have elucidated their additive and synergistic capability. Since AFB_1 and FB_1 commonly co-contaminate foods, any therapeutic approach that could mitigate both mycotoxins would be highly attractive and more cost-effective than a combination of approaches. In particular, populations most at risk for exposure to both mycotoxins also suffer from food insecurity and poor economic conditions. Thus, a remediation strategy for such communities must function to reclaim contaminated foods in their entirety and cause minimal interference to daily life. UPSN is from the family of smectites that has been shown to sorb AFB_1 *in vitro* and in numerous animal and human models, thereby decreasing biomarkers of exposure in the urine and blood and protecting animals from toxic endpoints (Beaver et al. 1990; Colvin et al. 1989; Edrington et al. 1996; Phillips et al. 1988; Pimpukdee et al. 2004). Recently,

in vitro analyses indicated that UPSN efficaciously bound FB_1 as well as mixtures of AFB_1 and FB_1 (Brown KA et al. 2012).

The objectives of this study were: 1) to determine whether a dual protection would be feasible in a mammalian gastrointestinal system and 2) to assess the difference in UPSN efficacy when a mixture of AFB_1 and FB_1 is present, as opposed to a single toxin exposure.

5.2 Materials and methods

5.2.1 Materials

High Performance Liquid Chromatography (HPLC) grade methanol and acetonitrile, as well as reagent grade hydrochloric acid, formic acid, sodium phosphate monobasic, sodium borate, sodium cyanide, dibasic potassium phosphate, and pH buffers (4.0, 7.0, and 10.0) were purchased from VWR (Atlanta, GA). *O*-phthaldialdehyde (OPA), AFM_1, and FB_1 analytical standards were purchased from Sigma-Aldrich (Saint Louis, MO). FB_1 specified for animal treatment was purchased from the PROMEC Unit of the South African Medical Research Council (Tygerbreg, South Africa). UPSN was obtained from Texas Enterosorbents (Bastrop, TX). Ultrapure deionized water (18.2 MΩ) was generated within the laboratory using an Elga™ automated filtration system (Woodridge, IL).

5.2.2 Animal housing, diet, and treatments

Five-week old male Fischer-344 rats were purchased from Harlan (Houston, TX) and allowed to acclimate for one week before being randomly divided into twelve treatment groups consisting of six animals per group (Table 19). Briefly, the treatment

groups included an absolute control, positive controls (dosed with AFB_1, FB_1 or a mixture of the two), and groups treated with a one-time toxin dose in addition to one of three levels of UPSN clay (0.25, 0.5, or 2% w/w feed consumption). Control animals (no UPSN) were maintained on a nutritionally complete powdered feed (Teklad rodent diet 8604, Harlan) and water *ad libitum*. Animals that were placed in the 0.25, 0.5, or 2% UPSN treatment groups received Teklad 8604 powdered feed homogenously mixed with UPSN at the respective percentages and water *ad libitum*. Animal maintenance, husbandry, and treatment protocols involving AFB_1 and FB_1 exposures were reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University. Following the one week acclimation period animals were gavaged with 0.125 mg AFB_1/kg b.w. and/or 25 mg FB_1/kg b.w. These levels were minimal doses to provide sufficient levels of urinary AFM_1 and FB_1 for quantitation (Robinson et al. 2012; Sarr et al. 1995). Aqueous gavage solutions administered to UPSN treatment groups also contained UPSN at an amount equivalent to one day's intake. Based on an average daily feed consumption of 20 g the doses were as follows: the 0.25% group was gavaged with 0.05 g, 0.5% were given 0.1 g, and 2% were given 0.4 g UPSN, respectively. Following gavage, animals were housed in metabolic cages (Nalgene^{TM}) and urine was collected at 12, 24, 36, 48, and 72 hr. Samples were stored at -20°C for AFM_1 and FB_1 biomarker analyses. Animals were euthanized after 72 hrs, blood samples collected, and serum stored at -20°C until AFB_1-albumin analysis. Liver and kidney weights were also recorded and expressed as a percentage of the total body weight.

5.2.3 Analysis of urinary AFM_1

AFM_1 concentrations in the urine were analyzed using immunoaffinity column purification using methods previously described in Section 2.2.3. However, here 1 ml of rat urine was adjusted to an acidic pH with 1.0 M ammonium formate (pH 4.5) and the volume increased to 10 ml with water. Quantification of AFM_1 was based on peak area and retention time as compared to external standards run between every 5 samples. The limit of detection for urinary AFM_1 using this method was 4.8 pg. Creatinine analysis was performed on all urine samples at the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) to adjust for variations in urine dilution.

Table 19. Study treatment groups

Treatment groups	0% UPSN	0.25% UPSN	0.5% UPSN	2.0% UPSN
0.125 mg AFB_1/kg b.w.	6	6	6	6
25 mg FB_1/kg b.w.	6	6	6	6
25 mg FB_1/kg b.w. + 0.125 mg AFB_1/kg b.w.	6	6	0	6
Absolute Control	6	0	0	0

5.2.4 Analysis of urinary FB_1

Urine samples from 3 animals in the same treatment group were pooled for analysis. Following centrifugation, a 300 µl aliquot of supernatant was loaded into a pre-equilibrated VICAM™ FumoniTest immunoaffinity column and passed through by gravity. After washing with 10 ml of PBS, FB_1 was eluted three times with 0.5 ml of 20% methanol in 10 mM hydrochloric acid directly onto a pre-conditioned Waters Oasis

HLB cartridge (3 cc/60 mg, 30 μm particle size). The HLB cartridge was sequentially washed with 2 ml water and 2 ml 30% aqueous methanol, and then eluted three times with 0.6 ml of 2% formic acid in methanol. Eluents were evaporated to dryness under nitrogen gas while incubated at 35°C. Dry residues were reconstituted with 150 μl of 50% aqueous methanol.

HPLC-fluorescence analysis was carried out on an Agilent 1100 liquid chromatography system (Agilent Technologies, Santa Clara, CA). FB_1 molecules were derivatized with *o*-phthaldialdehyde (OPA) prior to fluorescence detection (Xu et al. 2010). To avoid degradation of the derivatives, an on-line automatic injector thoroughly mixed 20 μl of OPA reagent with the sample for one minute immediately prior to each injection. Chromatographic separations were performed on a Luna C18 (2) column (Phenomenex Torrance, CA; 5 μm particle size, 250 x 4.6 mm), maintained at 35°C. Molecules were resolved with a linear gradient starting from 0.1 M sodium phosphate monobasic (pH 3.4): methanol (35/65, v/v) to 0.1 M sodium phosphate monobasic (pH 3.4): methanol (20/80, v/v) over 13 min, with a flow rate of 1.0 ml/min and injection volume of 100 μl. The OPA derivative was monitored at an emission wavelength of 440 nm and excitation wavelength of 330 nm. The limit of detection for this method was 20 pg.

5.2.5 Analysis of serum AFB_1-albumin adduct

Serum from all AFB_1 and AFB_1/FB_1 treatment animals was assessed for AFB_1-albumin levels 72 hr following gavage. The methodology to complete this analysis was adopted as previously reported (Qian G et al. 2013b; Riley et al. 1994). Briefly, 150 μL

of serum was digested with pronase (pronase:total protein, 1:4, w:w) for 3 hr at 37°C. The digest was further purified by a solid phase cartridge, evaporated, reconstituted, and injected into an Agilent 1200 HPLC system for quantification by fluorescence signal. Excitation and emission wavelengths were 405 and 470 nm, respectively. The limit of detection was 0.4 pg/mg albumin. Serum AFB_1-albumin adduct concentrations were adjusted by total serum albumin content.

5.2.6 Statistical analysis

Serum levels of AFB_1-albumin adduct and urinary AFM_1 and FB_1 are expressed as mean ± standard deviation (SD). Data was not normally distributed and was therefore log transformed for statistical analysis. Comparisons of these parameters between treated groups and the control group were conducted using one-way analysis of variance (ANOVA) followed by a student's t-test to compare between each of the 12 treatment groups. A p-value of less than 0.05 was considered statistically significant.

5.3 Results

5.3.1 Organ and body weights

Relative weight gain over the acclimation and treatment periods of the current study were not significantly different between absolute control and treated animals. Average weight gained during the acclimation period was 30.1 g. Animals weighed on average 145.3 g, 72 hr following gavage. Comparisons of organs between treatment groups were conducted with the somatic indices for each organ. There were no significant differences observed between treatment groups for liver or kidney, the two primary target organs known to be affected by FB_1 and AFB_1.

Average percent body weight values for liver and kidney were 4.77% ± 0.24 and 0.46% ± 0.04, respectively (data not shown).

5.3.2 Urinary AFM_1 levels in AFB_1 treatment groups

Mean urinary AFM_1 was calculated based on six rats per treatment group. Absolute control animals receiving an equal volume (0.75 ml) gavage of ultra-pure water did not have detectable AFM_1 metabolites in the urine at any of the collection time points. Excretion of AFM_1 peaked between 12 and 24 hr following AFB_1 treatment. Five out of six rats were completely void of AFM_1 by the 36 hr timepoint (Figure 15A). The positive control group displayed the highest average concentration of AFM_1 (201 ng/mg creatinine) at the 12 hr time point. Comparisons of AFM_1 levels between UPSN treatment groups and positive controls showed a marked decrease in overall excretion of AFM_1 metabolites. UPSN treatment reduced AFM_1 biomarkers in a dose-dependent manner with the largest reduction observed in the 2% treatment group. UPSN at 0.25% reduced AFM_1 excretion by 88%, 94%, and 85% at 12, 24, and 36 hr time points, respectively (Figure 15B). Reduction of biomarkers in the 0.5% UPSN treatment group were 91%, 96%, and 85% at 12, 24, and 36 hr time points (Figure 15B). AFM_1 levels in the 2% UPSN treatment group were reduced by 97%, 99%, and 58% at 12, 24, and 36 hr, respectively. The decrease in AFM_1 observed in the UPSN treatment groups was highly significant ($p<0.001$) at 12 and 24 hr. The 0.25% and 0.5% UPSN groups were also significantly different from the 2% UPSN group ($p=0.0045$ and 0.0190, respectively), but were not significantly different from each other (Figure 15A).

5.3.3 Urinary AFM_1 levels in AFB_1/FB_1 treatment groups

AFM_1 excretion patterns were different between AFB_1 treatment groups and AFB_1/FB_1 mixture groups. AFM_1 excretion by the co-exposure group peaked at 12 hr and was almost undetectable by 24 hr. The highest average AFM_1 concentration in the urine was 118 ng/mg creatinine at 12 hr in animals dosed with the AFB_1/FB_1 mixture (Figure 15C). This value is approximately 2 times lower than the average concentration in animals dosed with AFB_1 alone. UPSN treatment dose-dependently reduced AFM_1 excretion in animals exposed to the AFB_1/FB_1 mixture. However, the percent reductions observed in the mixture groups were lower than those in the AFB_1 control groups. Compared to AFB_1/FB_1 positive controls, the 0.25% UPSN group exhibited AFM_1 decreases of 66% and 32% at 12 and 24 hr, respectively. At the same time points, 0.5% UPSN reduced AFM_1 by 88% and 43%, while 2% UPSN reduced levels by 95% and 76% (Figure 15D). AFM_1 levels were non-detectable in any groups treated with UPSN by the 36 hr timepoint. AFM_1 excretion levels observed during UPSN inclusion were significantly different from the AFB_1/FB_1 control group for the 0.5% and 2% UPSN only at the 12 hour time point ($p=0.0124$ and 0.0016, respectively). The 0.25% UPSN group did not excrete significantly lower AFM_1 than the AFB_1/FB_1 controls at 12 hr, and excreted levels significantly higher than the 2% UPSN group ($p=0.0072$) (Figure 15C). Although the UPSN treated groups had lower AFM_1 output at 24 hr than the control group, the difference was not statistically significant.

5.3.4 Urinary FB_1 levels in FB_1 treatment groups

Urine samples were also analyzed for FB_1 with HPLC-coupled fluorescence detection. Mean FB_1 levels were calculated from six animals per treatment group. Absolute control animals did not have detectable levels of FB_1 present in their urine at any time point assessed. As expected, the highest levels of FB_1 excretion occurred 12 hr post-gavage, decreasing rapidly, and reaching non-detectable levels by 36 hr (Figure 16A). At 12 hr, mean urinary excretion for animals dosed with FB_1 alone was 722 ng/mg creatinine. Addition of UPSN clay at the 2% w/w level significantly decreased the amount of FB_1 excreted at 12 and 24 hr ($p=0.0437$ and 0.0150, respectively). Although not significant, 0.25% UPSN decreased overall excretion of FB_1 as well. Mean levels of FB_1 excreted by the 0.25% and 2% UPSN groups at 12 hr were 425 and 384 ng/mg creatinine, respectively, and 177 and 281 ng/mg creatinine at 24 hr. Overall reduction of the FB_1 biomarker for the 0.25% UPSN inclusion group was 41% at 12 hr and 61% at 24 hr, compared to the positive control group. UPSN inclusion at the 2% level reduced urinary FB_1 by 80% and 98% at 12 and 24 hr (Figure 16B).

5.3.5 Urinary FB_1 levels in AFB_1/FB_1 treatment groups

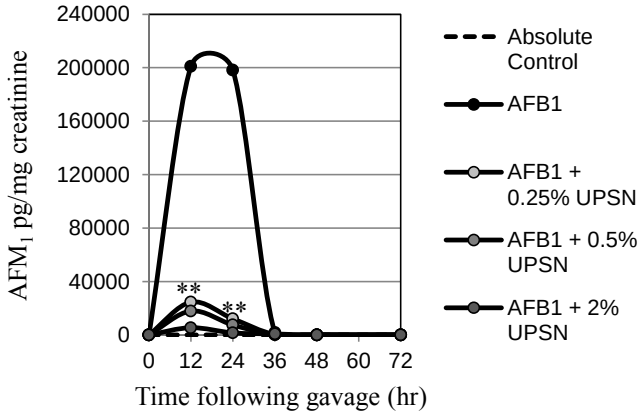
Mean urinary levels of FB_1 were 453 and 78 ng/mg creatinine at 12 and 24 hr post-gavage, respectively, in the AFB_1/FB_1 co-treated positive control samples. A dose-dependent reduction in urinary FB_1 was observed for the UPSN treated AFB_1/FB_1 groups, with the 2% UPSN group exhibiting the largest difference from the positive control group (Figure 16C). The FB_1 biomarker in the 0.25% UPSN group was reduced by 24 and 27% at 12 and 24 hr after gavage and by 40 and 50% for the 2% UPSN group,

respectively (Figure 16D). Reduction in FB_1 was significant in the 2% UPSN group at 12 hr (p=0.0137), but not at 24 hr (p=0.1452). The reduction of urinary FB_1 excretion observed in the 0.25% UPSN group was not statistically significant, with p=0.0910.

5.3.6 Area under the curve (AUC) calculations

Figure 17 shows the differences in kinetics for total AFM_1 and FB_1 excretion in the urine by calculations of area under the curve (AUC) for each treatment group. Based on AUC, approximatley 75% of the total AFM_1 excreted was detected within the first 24 hr after AFB_1 exposure in the positive control group. The 0.25% UPSN group excreted the equivalent of 9% of the total AFM_1 that was excreted by the AFB_1 treated positive control group (Figure 17A). Based on the total AUC, the 0.5 and 2% UPSN groups excreted the equivalent of 6 and 2% of the amount of AFM_1 excreted by the AFB_1 treated positive control, respectively. The mean excretion of AFM_1 biomarkers was significantly different (p=0.0002) between animals dosed with AFB_1 alone and animals receiving both AFB_1 and FB_1. The AUC value for AFM_1 excretion in the AFB_1/FB_1 control animals was approximately 3 times smaller than the AUC for the AFB_1 control animals (Figure 14A). Based on AUC values, the 0.25%, 0.5%, and 2% UPSN groups excreted the equivalent of 37%, 16%, and 7% of the AFM_1 excreted by the AFB_1/FB_1 positive controls.

A.

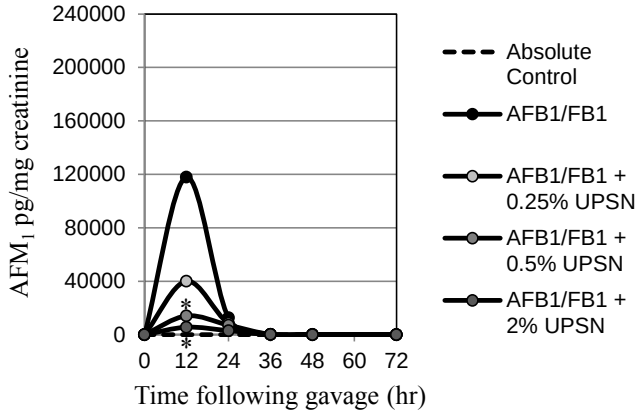


B.

Percent Reduction of AFM_1 for AFB_1 treated groups

Time point	12 hr	24 hr	36 hr
0.25% UPSN	88	94	85
0.5% UPSN	91	96	85
2% UPSN	97	99	58

C.



D.

Percent Reduction of AFM_1 for AFB_1/FB_1 treated groups

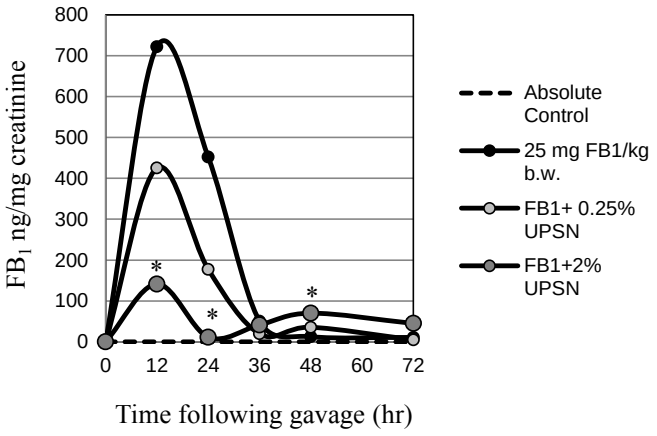
Time point	12 hr	24 hr	36 hr
0.25% UPSN	66	32	ND
0.5% UPSN	88	43	ND
2% UPSN	95	76	ND

Figure 15. Mean excretion pattern of AFM_1. A) 0.125 mg AFB_1/kg b.w. treated groups with 0, 0.25, 0.5, and 2% UPSN. B) Table of percent reduction between AFB_1 treated positive control group (no clay) and each AFB_1/UPSN group at time points 12, 24, and 36 hr following gavage. C) AFB_1/FB_1 mixture groups with 0, 0.25, 0.5, and 2% UPSN. D) Table of percent reduction between AFB_1/FB_1 treated positive control group (no clay) and each AFB_1/FB_1/UPSN group at time points 12, 24, and 36 hr following gavage. * Indicates a significant difference between the positive control group and the UPSN treated group at a specific time point ($p<0.01$). ** Indicates a significant difference between the positive control group and ALL UPSN groups at a specific time point ($p<0.01$).

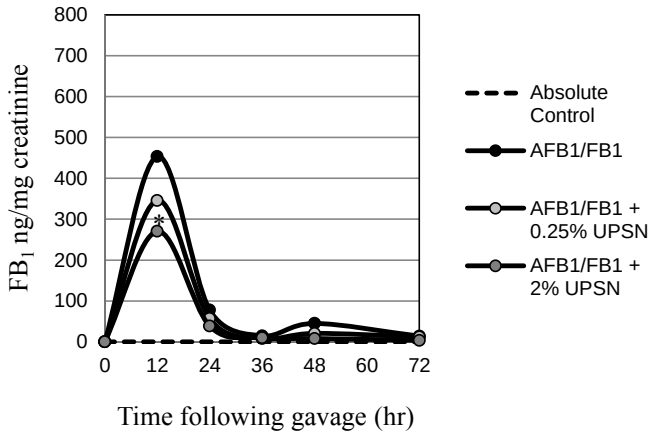
Figure 15. Mean excretion pattern of AFM_1. A) 0.125 mg AFB_1/kg b.w. treated groups with 0, 0.25, 0.5, and 2% UPSN. B) Table of percent reduction between AFB_1 treated positive control group (no clay) and each AFB_1/UPSN group at time points 12, 24, and 36 hr following gavage. C) AFB_1/FB_1 mixture groups with 0, 0.25, 0.5, and 2% UPSN. D) Table of percent reduction between AFB_1/FB_1 treated positive control group (no clay) and each AFB_1/FB_1/UPSN group at time points 12, 24, and 36 hr following gavage. * Indicates a significant difference between the positive control group and the UPSN treated group at a specific time point ($p<0.01$). ** Indicates a significant difference between the positive control group and ALL UPSN groups at a specific time point ($p<0.01$).

Approximately 76% of the total FB_1 excreted by the FB_1 treated positive control group was excreted within the first 24 hr after gavage. Based on the AUC calculations, the 0.25% UPSN group excreted the equivalent of 54% of the total FB_1 excreted by the FB_1 treated control group. The 2% UPSN group excreted the equivalent of 27% of the total FB_1 excreted by the FB_1 treated control group (Figure 17B). Similar to the change in kinetics of AFM_1 excretion after AFB_1/FB_1 co-exposure, total FB_1 excreted in AFB_1/FB_1 combination groups was significantly lower than total FB_1 excreted in the FB_1 treated controls ($p=0.0173$). Total AUC values indicated an approximate two-fold reduction in the amount of FB_1 excreted by the AFB_1/FB_1 combination group compared to the positive control group. Based on the AUC calculations, the 0.25% and 2% UPSN groups excreted the equivalent of 72 and 53% of the total FB_1 excreted by the AFB_1/FB_1 positive controls (Figure 17B). UPSN treatment was less effective in reducing AFM_1 and FB_1 urinary biomarkers after AFB_1/FB_1 co-exposures than after exposure to either AFB_1 or FB_1 alone.

A.



C.



B.

Percent Reduction of FB_1 for FB_1 treated groups

Time point	12 hr	24 hr	36 hr
0.25% UPSN	41	61	ND
2% UPSN	80	97	ND

D.

Percent Reduction of FB_1 for AFB_1/FB_1 treated groups

Time point	12 hr	24 hr	36 hr
0.25% UPSN	24	27	ND
2% UPSN	40	50	ND

Figure 16. Mean excretion pattern of FB_1. A) 25 mg FB_1/kg b.w. treated groups with 0, 0.25, and 2% UPSN. B) Table of percent reduction between FB_1 treated positive control group (no clay) and each FB_1/UPSN group at time points 12 and 24 hr following gavage. C) AFB_1/FB_1 mixture groups with 0, 0.25, and 2% UPSN. D) Table of percent reduction between AFB_1/FB_1 treated positive control group (no clay) and each AFB_1/FB_1/UPSN group at time points 12 and 24 hr following gavage. * Indicates a significant difference between the positive control group and the UPSN treated group at a specific time point ($p<0.05$).

Figure 16. Mean excretion pattern of FB_1. A) 25 mg FB_1/kg b.w. treated groups with 0, 0.25, and 2% UPSN. B) Table of percent reduction between FB_1 treated positive control group (no clay) and each FB_1/UPSN group at time points 12 and 24 hr following gavage. C) AFB_1/FB_1 mixture groups with 0, 0.25, and 2% UPSN. D) Table of percent reduction between AFB_1/FB_1 treated positive control group (no clay) and each AFB_1/FB_1/UPSN group at time points 12 and 24 hr following gavage. * Indicates a significant difference between the positive control group and the UPSN treated group at a specific time point ($p<0.05$).

5.3.7 AFB_1-albumin analysis

Analysis of AFB_1-albumin adducts in the serum was conducted following euthanasia (72 hr after gavage). Means and standard deviations were calculated based on three animals per group. Compared to positive control groups, all three UPSN concentrations significantly reduced AFB_1-albumin adduct levels (all p-values<0.0001). UPSN concentrations of 0.25% and 0.5% were also significantly different (p<0.0001) compared to the 2% group, but not to each other (Figure 18A). The mean AFB_1-albumin concentration was 632 pg/mg albumin for the positive control group (AFB_1 treatment alone), whereas serum from the 0.25%, 0.5%, and 2% UPSN groups contained 101, 104, and 36 pg/mg albumin, respectively. The percent reduction in AFB_1-albumin levels by UPSN treatment group are shown in Figure 18B. Animals that were gavaged with a combination of AFB_1/FB_1 had significantly higher AFB_1-albumin levels (p=0.0061) than the AFB_1-treated controls. However, UPSN clay was still capable of significanlty decreasing the AFB_1-albumin biomarker in a dose-dependent manner (Figure 18A). In the AFB_1/FB_1 combination group, the mean AFB_1-albumin level was 1,112 pg/mg albumin.The adduct concentrations in the 0.25%, 0.5%, and 2% UPSN groups co-exposed to AFB_1/FB_1 were significantly different from each other (242, 126, and 37 pg/mg albumin, respectively).

5.4 Discussion

Previous isothermal data from our laboratory indicated that NovaSil clay (a calcium montmorillonite) can tightly sorb AFB_1 and reduce the bioavailability of AFB_1 in the gastrointestinal tract of animals and humans (Phillips et al. 1995, 2008).

Importantly, clay treatment resulted in lower levels of well-established short and long-term biomarkers of AF exposure in the urine and serum, i.e. AFM_1 and AFB_1-albumin adducts (Wang P et al. 2008). Recent studies demonstrated that NovaSil clay also reduced biomarker levels of FB_1 in rats and humans (Robinson et al. 2012). A uniform particle size NovaSil clay (UPSN) was produced to maintain purity and consistency from batch to batch for use in human populations. In preliminary work, UPSN was shown to retain the binding properties of the parent product (NovaSil) (Marroquin-Cardona et al. 2011). Binding analysis performed with heat-collapsed UPSN suggests that AFB_1 and FB_1 binding is saturable and occurs largely within the interlayer regions of the clay (Brown KA et al. 2012; Grant and Phillips 1998) providing preliminary evidence that both mycotoxins may compete for the same binding sites on surfaces of UPSN.

In the present study, inclusion of UPSN montmorillonite clay during AF exposure decreased the excretion of AFM_1 in the urine and the production of the AFB_1-albumin adduct in the serum of rats. The overall excretion of AFM_1 was similar to that reported in a study utilizing Fischer-344 rats in which the majority of AFM_1 excreted in the urine occurred within the first 12 hr following gavage of animals with AFB_1.

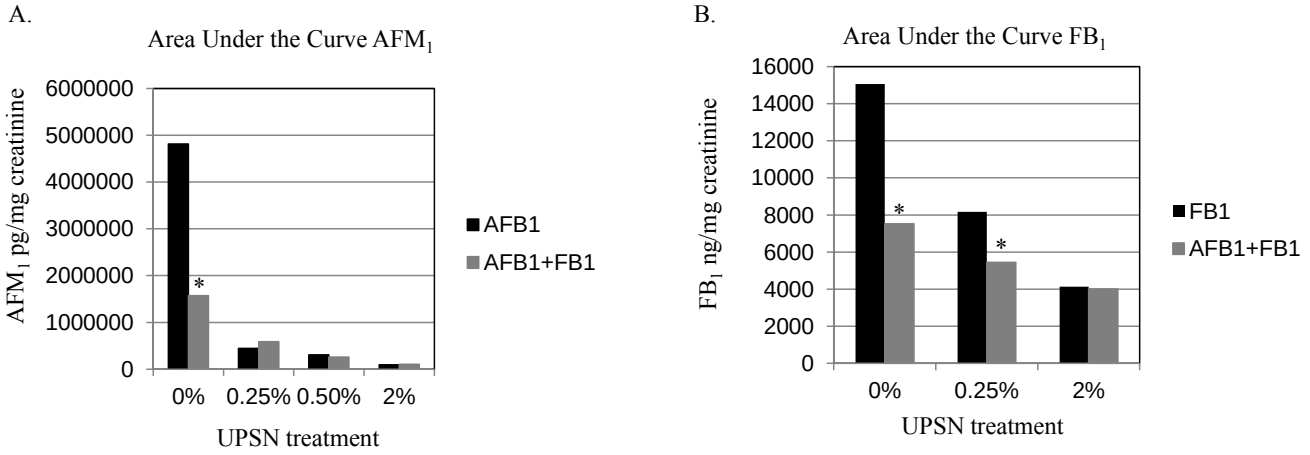


Figure 17. Area under the curve for AFM_1 and FB_1 excretion. A) Area under the curve (AUC) for toxicokinetic excretion of AFM_1 in urine. Black bars are indicative of total AUC for rats treated with 0.125 mg AFB_1/kg b.w. and 0, 0.25, 0.5, or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB_1/FB_1 mixture and 0, 0.25, 0.5, or 2% UPSN. B) AUC for pharmacokinetic excretion of FB_1 in urine. Black bars are indicative of total AUC for rats treated with 25 mg FB_1/kg b.w. and 0, 0.25, 0.5, or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB_1/FB_1 mixture and 0, 0.25, 0.5, or 2% UPSN. * Indicates a significant difference between the single toxin treatment and the mixture treatment with the same inclusion percent of UPSN ($p<0.01$).

Figure 17. Area under the curve for AFM_1 and FB_1 excretion. A) Area under the curve (AUC) for toxicokinetic excretion of AFM_1 in urine. Black bars are indicative of total AUC for rats treated with 0.125 mg AFB_1/kg b.w. and 0, 0.25, 0.5, or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB_1/FB_1 mixture and 0, 0.25, 0.5, or 2% UPSN. B) AUC for pharmacokinetic excretion of FB_1 in urine. Black bars are indicative of total AUC for rats treated with 25 mg FB_1/kg b.w. and 0, 0.25, 0.5, or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB_1/FB_1 mixture and 0, 0.25, 0.5, or 2% UPSN. * Indicates a significant difference between the single toxin treatment and the mixture treatment with the same inclusion percent of UPSN ($p<0.01$).

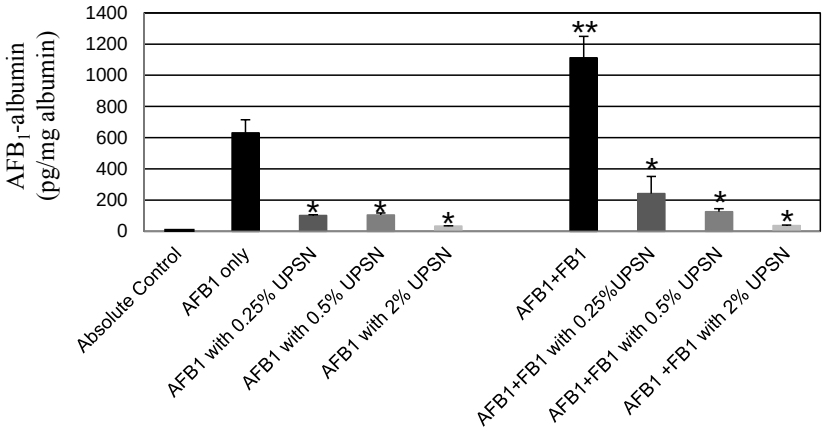
Inclusion of 0.5% of a calcium montmorillonite resulted in a reduction of AFM_1 excretion by 95%, while in the current study, 0.5% UPSN inclusion resulted in a 94% total reduction in AFM_1 (Sarr et al. 1995). A study utilizing dogs, demonstrated that calcium montmorillonite treatment, via food inclusion, following AFB_1 gavage could lower urinary excretion of AFM_1 by as much as 65% (Bingham et al. 2004). Species differences in absorption, metabolism, length of digestion, clay dose dissemination, and treatment regimen could all possibly account for the differences in efficacy seen between these studies. While dogs will consume an entirety of a meal in one sitting, this is not typically the case in rodents, especially following gavage, which can be a stressful procedure. Thus, it was deemed necessary to include UPSN in the gavage solution for the rodent model to ensure adequate time in the GI tract to interact with AFB_1 before adsorption into the vascular system. The difference in binding efficacy obtained in the current study could be ascribed to the route of exposure (i.e., gavage vs. dietary) and other physiological conditions prevailing in the gut of rodents.

Although extensive research focusing on the binding capacity of calcium montmorillonite for AF has been conducted, it is only recently that its sorption capability for FB has been addressed. Robinson et al. (2012) demonstrated a significant decrease in FB biomarkers of exposure following treatment with clay in both rats and humans. In the rodent model given 2% clay, FB_1 excretion in the urine was reduced by 20% at 24 hr post-gavage and 50% at 48 hr post-gavage (Robinson et al. 2012). In the current study, the reduction of FB_1 biomarkers following inclusion of UPSN at 2% was higher (97%) after 24 hr. The protocols for these two studies were similar; however, the current study

collected urine samples at more frequent time intervals and the samples were pooled (3 animals/analysis) within treatment groups. It is also important to note, that in this study a refined form of calcium montmorillonite (UPSN) was used which was not the same clay used in the study by Robinson et al. (2012). While there is no evidence that UPSN would have a higher capacity to bind FB, the isothermal analysis has not been conducted to verify this hypothesis, and thus could still be considered a factor in the higher reduction of urinary metabolites.

The present study verifies the results reported for other rodent based metabolic studies indicating that treatment with UPSN can reduce the excretion of AFM_1 and FB_1 in the urine following exposure to the mycotoxins separately. Since AFB_1 and FB_1 can co-occur under natural conditions (Almeida et al. 2012; Kimanya et al. 2008; Sun G et al. 2011), this could enhance risk due to possible synergistic and/or additive effects. The mixture of AFB_1 and FB_1 resulted in a slightly lowered efficacy of UPSN in reducing these mycotoxins. Based on AUC calculations, the 2% UPSN group reduced the total amount of AFM_1 excreted by 98% in the AFB_1 treated group alone, and 93% in the AFB_1/FB_1 treated group. AUC values indicated that 2% UPSN decreased total FB excretion by 73% in the FB_1 treated group and only reduced by 47% in the AFB_1/FB_1 mixture. The reduced efficacy of UPSN binding observed in the mixture groups could be an indicator of competition for binding sites on the clay. It is evident from this data that although UPSN can bind both AFB_1 and FB_1, it will preferentially bind AFB_1

A.



B.

Percent reduction in AFB_1-albumin levels

Treatment	AFB_1	AFB_1+FB_1
0.25% UPSN	84	78
0.5% UPSN	83	89
2% UPSN	95	97

Figure 18. Serum AFB_1-albumin concentrations. A) Mean serum AFB_1-albumin levels for the AFB_1 and AFB_1/FB_1 treated groups. *Indicates a significant difference between positive control group and UPSN treated group ($p<0.001$). ** Indicates a significant difference between the AFB_1 and AFB_1/FB_1 group ($p<0.05$). B) Table of percent reduction between positive control group (no clay) and each UPSN group.

Figure 18. Serum AFB_1-albumin concentrations. A) Mean serum AFB_1-albumin levels for the AFB_1 and AFB_1/FB_1 treated groups. *Indicates a significant difference between positive control group and UPSN treated group ($p<0.001$). ** Indicates a significant difference between the AFB_1 and AFB_1/FB_1 group ($p<0.05$). B) Table of percent reduction between positive control group (no clay) and each UPSN group.

during co-exposures. This hypothesis is further supported by the fact that there were more molecules of FB ($3.46x10^{-5}$ mole/kg) available for binding than molecules of AF ($4.01x10^{-7}$ mole/kg). Inclusion of 2% UPSN clay could protect humans or animals against the toxic effects of AFB_1 when there is a mixture of mycotoxins present, however it may not be sufficient to completely eliminate the effects from FB_1 exposure. In contrast to AFB_1, a non-genotoxic threshold exists for FB_1, (JECFA 2001) suggesting that the impaired adsorption may well reduce levels *in vivo* below that which would result in any adverse biological effects. This is of relevance in populations where exposure levels of FB_1 exceed the provisional tolerable daily intake (PMTDI) proposed by JECFA (2001), and UPSN intervention could play an important role in reducing the risk of exposure. More research, however, needs to be conducted to determine a sufficient level of UPSN inclusion in the diet that could positively impact exposure and relevant physiological and/or biological endpoints for combined AFB_1 and FB_1 toxicity.

Apart from the effect of UPSN, the amount of AFM_1 and FB_1 excretion in the urine was significantly lower when the toxins were dosed together than when either was dosed alone in control groups. Since AFB_1 is more toxic than FB_1 the relative reduction in their absorption from the gut should also be considered. In addition, it is know that AFB_1 is absorbed at a high rate, whilst FB_1 is poorly absorbed ranging from 1 to 6% in non-ruminants (Gan et al. 1988; Scholl P et al. 1996). Studies, utilizing trans-epithelial electrical potential suggest that mycotoxins modulate the Na^+ co-transport involved in sugar and amino acid transport carrier systems (Grenier and Appelgate 2013). It is not known whether the uptake of AFB_1 and FB_1 also co-interact via these mechanisms,

although the current study implies that they countered each other's' absorption from the gut. The modulating role of FB_1 on lipid metabolism with the disruption of sphingolipid, phospholipid, cholesterol, and fatty metabolism has been reported, resulting in toxic effects including an increase in oxidative damage to different cellular constituents including the cellular membrane (Abel and Gelderblom 1998; Gelderblom et al. 2001; Riley et al. 2001). These different adverse biological parameters are likely to disrupt membrane integrity, which led to the hypothesis that the intestine is a possible target for FB toxicity (Bouhet and Oswald 2007). This could explain the poor absorption of FB_1 and, in the current study, the disrupted absorption of AFB_1, which provides interesting scenarios regarding the co-exposure of these carcinogens. Both the *in vivo* interactive liver AFB_1/FB_1 models utilize AFB_1 as the cancer initiating treatment with FB_1 as the promoter in a separate treatment regimen. The present study implies that the combined treatment is likely to reduce the cancer promoting properties of FB_1.

Although the urinary biomarkers were reduced when AFB_1 and FB_1 were co-treated, the blood AFB_1-albumin biomarker was increased in the presence of FB_1. Multiple pathways of detoxification and activation occur during metabolism of AFB_1 involving many different cytochrome P-450 enzymes (CYP-450). In particular, the AFM_1 and AFB_1-albumin products are considered to be involved in two separate pathways (detoxification and activation, respectively) but can be products of the same enzymes (Massey et al. 1995). In rats, the formation of AFM_1 is catalyzed predominantly by CYP1A1 and 1A2, while the production of the AFB_1 8,9-epoxide occurs through catalysis with CYP1A2, CYP2A3, CYP2B7, CYP3A3, and CYP3A4

with the latter isoform having the highest affinity for AFB_1 (Massey et al. 1995). A higher level of the AFB_1-albumin adduct suggests an increased production of the most toxic and carcinogenic metabolite, AFB_1 8,9-epoxide in the presence of FB_1. In earlier work, it was shown that FB_1 induced the activity of CYP1A1, 3A1 and 4A1 in rats following a 6 day intraperitoneal treatment (Martinez-Larranaga et al. 1996). This induction may be responsible for the higher level of the AFB_1-albumin adduct observed in this study. Thus, the carcinogenic potency of AFB_1 is likely to increase in the presence of FB_1. More studies are warranted to support this conclusion and the consequences of combinations of toxins in the diet.

Results from this study provide evidence for an economical and sustainable intervention to reduce exposure to both AF and FB from contaminated diets. Interesting interactive effects were noticed related to the combined treatment of the mycotoxins in the absence of UPSN, resulting in a 50% and 60% reduction in FB_1 and AFB_1 adsorption. The metabolic conversion of AFB_1 could also be altered resulting in an increased conversion into the 8,9 epoxide and AFM_1 presumably due to the selective induction of CYP isoforms by FB_1. While more research is warranted to find an optimal inclusion level for UPSN, the prospect of utilizing this clay as a binder for both toxins is promising. This could facilitate important applications to selectively reduce levels below the carcinogenic thresholds for initiation and promotion of hepatocellular carcinoma.

6. SUMMARY

Chronic exposure to dietary AFs remains a public health concern in communities consuming large quantities of maize and/or peanut based foods. AF is a known carcinogen primarily targeting the liver. In addition to HBV infection, AF has been implicated as a major risk factor for the development of HCC in Western Africa and Southeast Asia. Importantly, HBV and AF co-exposure causes a synergistic potency in HCC risk (calculated to be approximately 30 times higher than with either factor alone). In addition, a recent risk assessment study estimated that AF-induced HCC (without HBV) accounted for 2,150 to 9,300 annual cases of HCC in Africa. Reports of acute incidences of aflatoxicosis, resulting in the deaths of 125 people in Kenya, have verified the common occurrence and high levels of AF contamination present in the developing world. Even the young are at risk for exposure from ingestion of contaminated breast milk and weaning foods.

The young of all species are more susceptible to the effects of AF than adults. AF exposure in the young of various animal species has resulted in growth inhibition and faltering. Associations of growth stunting and AF consumption in children in Benin have created interest in interventions to reduce the negative effects on child health. Research from Ghana, in particular, has indicated a high frequency and level of exposure to AF in a farming community located in the Ashanti region. Similarly, a recent estimate of dietary AF consumption in Ghana was calculated to be one of the highest in Africa, due to the high maize content of the diet (Shephard et al. 2008). The burden of AF exposure from the diet in developing countries is a significant problem in the young (and the

vulnerable). Moreover, a variety of diverse intervention strategies have not proven to be sustainable and practical. Given the complicated cultural, economic, and logistical aspects that need to be considered when implementing an intervention, a comprehensive survey was conducted in different regions of Ghana to determine risk factors for exposure in this particular country.

An initial objective of our work was to assess AF exposure through biomarker analysis from urine samples across different regions of Ghana, which have different dietary habits due to cultural, economic, and demographic circumstances. Participant urine samples tested positive for a short-term biomarker (AFM_1) in 40% of the population. This level was significantly lower than observed in samples from the Ejura-Sekyedumase district in the Ashanti region of Ghana (Obuseh et al. 2010). It is important to note that the AFM1 biomarker can fluctuate with changes in the diet, and only predicts AF exposures from day to day due to its short half-life. The Greater Accra and Central regions of Ghana had the highest percentages of their populations above the median level of AFM_1, and should be considered as areas of importance for interventions. The most notable commonality between these two regions is location; both regions are coastal areas and have the highest population density of all the regions. Other socio-economic factors, such as gender, age, level of education, and number of persons in a household did not appear to affect AF load. Additionally, food preparation practices had no effect on overall AF exposure in the participants from this study. Thus, education and food sorting/washing, although important for other environmental and foodborne contaminants, may need complimentary practices to significantly reduce AF

contamination of foods in Ghana. As suspected, consumption of maize was a significant risk factor in AFM_1 excretion. More than 60% of the participants reported eating maize or maize-based products every day. This was higher than peanuts or millet, two other important crops that are susceptible to AF contamination. Thus, intervention strategies in Ghana should be focused on maize crops and maize-based foods. An appropriate intervention should be effective across all economic classes, genders, and age groups, and needs to compliment food preparation practices already present in the area. Use of an AF sorbent like calcium montmorillonite clay could prevent bioavailability of the foodborne toxin that is still present following sorting and washing of contaminated grains.

In numerous animals, NovaSil clay has been shown to be highly effective in mitigating the toxic effects of AF exposure from the diet. A three-month intervention trial in humans, demonstrated the efficacy of NovaSil to reduce the bioavailability of AFs from contaminated food (Wang P et al. 2008). Use of biomarkers of exposure to determine the efficacy of intervention trials has long been the standard for AF research due to the latent onset of AF related disease. However, previous interventions have indicated that it takes at least one-month to observe significant decreases in biomarker levels following initiation of treatments. Costly and time consuming trials are not plausible for application in the vulnerable, i.e. infants, children, and pregnant females due to safety and dosimetry concerns for new products and the requirement for short-term, Phase I studies prior to long-term interventions. In other work, a short-term (two-week) cross-over trial was conducted to evaluate the efficacy of the AFM_1 biomarker

and the significance of clay (UPSN) delivery in food, instead of capsules. Although AFM_1 excretion is positively correlated with AFB_1 intake, the day to day variability of this biomarker complicates statistical analysis. However, the cross-over study indicated that collecting daily urine samples and pooling the data over five days was effective in showing significant reductions of AF exposure in a preliminary trial. Urinary AFM_1 was reduced by 55% in the UPSN (refined NovaSil) treated group when compared to the placebo within five days. Interestingly, the cross-over design clearly indicated the switch in treatment groups using the AFM_1 biomarker. Future work to alleviate aflatoxicosis in children during periods of severe drought could be conducted over short periods of time with the use of daily AFM_1 biomarkers to indicate individual exposure and efficacy of the clay-based therapy. This would significantly reduce the risk of utilizing experimental treatments for children when dosimetry has yet to be determined in a vulnerable population. It is also important to note that this work also provided evidence that UPSN can significantly reduce the bioavailability of AF within five days. Thus, it is the only intervention strategy yet reported that has the potential to decrease morbidity and mortality in young humans and animals during AF outbreaks.

Safety and efficacy of reducing AF-induced biological endpoints has also been demonstrated in multiple animal models. Afriyie-Gyawu et al. (2008) reported no adverse events related to NovaSil consumption in adults over a three-month period. Following treatment with NovaSil for three months, serum levels nutrient and non-nutrient minerals were measured, with NovaSil having a dose-dependent effect on strontium (Sr) only. In further work, a Phase I safety evaluation of UPSN in children was

conducted to determine dosimetry and potential adverse events in order to translate this technology to rural communities in Ghana. Malnutrition and growth faltering result in more than 2 million deaths in children per year; importantly, the introduction of nutritional supplements in Africa has not had a significant impact on these outcomes. Many supplements utilized in Ghana, particularly for the very poor, are homemade porridges that contain high contents of maize and peanuts. In a survey of 36 households, samples of homemade supplements from the Ejura-Sekyedumase district tested positive for AF, with more than 80% of them above the FDA action level of 20 ppb (and one as high as 500 ppb). However, discontinuing these supplements could lead to increased mortality and morbidity in children from malnutrition. Utilization of UPSN in such foods could significantly decrease the effects from AF while maintaining the nutritional benefits. At our study site in Ejura, treatment of participants (3-9 years of age) with either 0.75, 1.5 g/day UPSN, or calcium carbonate (placebo) did not result in any severe adverse events. The most common complaint was vomiting; however the majority of those cases were diagnosed with malaria. Hematology and serum biochemistry parameters did not show a significant difference between UPSN and placebo groups. Only magnesium (Mg) showed signs of a dose-dependent decrease following the two-week ingestion of UPSN. Those participants consuming the high-dose of UPSN also had a significant decrease in AFM_1 excretion when compared to the placebo group. Results from this work are promising for the future use of UPSN in nutritional supplements destined for children. Work needs to be conducted to verify the reduction of Mg in the

serum and further determine a minimal effective dose that would be safe for use in children.

The continued work in Ejura has recently indicated that this population is co-exposed to another important mycotoxin, FB. The homemade nutritional supplements from this area all contained FB and 56% of urine samples from adults tested positive for an FB_1 biomarker (Robinson et al. 2012). FB, like AF, is known to highly contaminate maize and initial work with both toxins demonstrated either an additive or synergistic effect in cellular toxicity and development of HCC in animal models. Therefore, an intervention that can alleviate both AF and FB from the diet would have a dual benefit to the population in Ejura-Seykedumase. In our work, NovaSil clay was effective, albeit to a much lesser extent than with AF, to reduce the FB_1 biomarker in the urine of rats and humans. However, the binding capacity was significantly diminished following dehydroxylation and denaturing of the interlayer space of the clay (Brown et al. 2013), similar to the effects seen with AF. Thus, it was hypothesized that both AF and FB were capable of binding to the interlayer surfaces of the montmorillonite and were competing for binding sites. In other work, a metabolic study in rats was undertaken to delineate efficacy of UPSN in an animal model when both AFB_1 and FB_1 are present. UPSN significantly reduced urinary biomarkers of both AFB_1 and FB_1 when dosed separately. Interestingly, when in combination, UPSN lost some of its efficacy for both mycotoxins, although it still appeared to bind AFB_1 preferentially over FB_1. This would indicate that UPSN can be effective in reducing the bioavailability of both mycotoxins; however

competition for binding sites may reduce its efficacy and should be considered in future intervention trials.

In summary, AF and FB exposure in Western Africa is seemingly unresolved due to food insecurity and climate. Although AF is known to contribute to the morbidity and mortality from HCC in the area, it is likely that its impact is even more significant on public health through its negative effects on immunity, growth, and nutrient utilization. Co-exposure to FB is likely to only exacerbate the issue, as it is known to cause cytotoxic and chronic inflammatory problems. Practical and effective intervention strategies focused on alleviating the exposure in poor communities and communities at high risk for both mycotoxins are critically needed. Inclusion of UPSN in the diets of such populations could be a sustainable approach to reduce the health effects of these mycotoxins in the vulnerable, thus positively impacting the health of humans and animals in the developing world.

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